



Nitrite-cured cooked pork products – Characterisation of antioxidative and antimicrobial activities

Pedersen, Sabine Tauber

Publication date:
2018

Document Version
Publisher's PDF, also known as Version of record

[Link back to DTU Orbit](#)

Citation (APA):
Pedersen, S. T. (2018). *Nitrite-cured cooked pork products – Characterisation of antioxidative and antimicrobial activities*. Technical University of Denmark.

General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Nitrite-cured cooked pork products

- Characterisation of antioxidative and antimicrobial activities



Sabrine Tauber Pedersen
PhD thesis
May 2018

Nitrite-cured cooked pork products

– Characterisation of antioxidative and antimicrobial activities

Sabrine Tauber Pedersen
PhD Thesis

Division of Food Technology
National Food Institute
Technical University of Denmark

May 2018

Title sheet

Title: Nitrite-cured cooked pork products – Characterisation of antioxidative and antimicrobial activities

Author: Sabine Tauber Pedersen

Affiliation: National Food Institute
Technical University of Denmark
Division of Food Technology
Research Group for Food Production Engineering

e-mail: stape@food.dtu.dk

Supervisors:

Flemming Jessen, Senior Researcher, PhD
National Food Institute
Technical University of Denmark
Division of Food Technology
Research Group for Food Production Engineering

Lene Duedahl-Olesen, Associate Professor, PhD
National Food Institute
Technical University of Denmark
Research Group for Analytical Food Chemistry

Anette Granly Koch, Technical Manager, PhD
Danish Meat Research Institute
Technological Institute
DK-2630 Taastrup, Denmark

Caroline P. Baron, Head of Laboratory, PhD
BIOFAC A/S
DK-2770 Kastrup, Denmark

Funding: This project was financially supported by Norma and Frode S. Jacobsens Fund, Danish Meat Research Institute and Technical University of Denmark

Preface

This thesis entitled “Nitrite-cured cooked pork products – Characterisation of antioxidative and antimicrobial activities” presents the work conducted as part of my PhD study and was submitted in order to meet the requirements for obtaining the PhD degree at the National Food Institute, Technical University of Denmark.

The work was conducted in the research groups of Food Production Engineering, Bioactives – Analysis and Application and the former Protein and Quality at the division of Food Technology, National Food Institute, Technical University of Denmark between the 15th of November 2014 and the 14th of May 2018. The chemical part of the experimental work was carried out at Technical University of Denmark, University of Copenhagen, Aalborg University and Aarhus University while the microbiological investigations were carried out by Danish Meat Research Institute. The project was supervised by Senior Researcher Flemming Jessen (National Food Institute, Technical University of Denmark) as main supervisor and Associate Professor Lene Duedahl-Olesen (National Food Institute, Technical University of Denmark), Caroline P. Baron (former Associate Professor at National Food Institute, Technical University of Denmark, currently – BIOFAC A/S) and Technical Manager Anette Granly Koch (Danish Meat Research Institute, Danish Technological Institute) as co-supervisors.

The project was kindly funded by the Norma and Frode S. Jacobsens Fund, Danish Meat Research Institute and Technical University of Denmark.

Acknowledgements

The past three and a half year has been an intense experience and I would like to thank the many people giving me the opportunity and strength to pursue a PhD.

First of all I would like to show gratitude to my DTU supervisors Flemming Jessen, Lene Duedahl-Olesen and Caroline P. Baron for their time and experienced supervision. Especially, thanks to Flemming for supervising me with great patience and supporting me wherever and whenever possible. A special thanks to Lene for her caring nature, sharing my passion for the quality of teaching and being a great traveling partner. Lastly to Caroline; I cannot express my appreciation for you always believing in me. I would also like to thank the people from Danish Meat Research Institute including Anette Granly Koch, Flemming Hansen and Anita Forslund for supervision and great assistance on all microbiological aspects in the project as well as procurement of sample material.

For great collaboration and sharing of expertise and connections in field of mass spectrometry much appreciation is also expressed to Post doc Cristian De Gobba and Associate Professor René Lametsch from University of Copenhagen and Senior Researcher Emøke Bendixen and Laboratory Technician Dorte Thomassen from Aarhus University. From the National Food Institute, Technical University of Denmark thanks to Professor Jørn Smedsgaard and Senior Researcher Henrik Lauritz Frandsen for offering their great experience and analytical minds for cracking of some of the challenges encountered in the project.

I would also like to thank my colleagues at the National Food Institute for contributing to creating a good working environment – thanks for your company, your support and the fun times we shared. Furthermore, I would like to thank the students I have had the privilege of supervising. It has been a great pleasure working with you and you have provided me with valuable contributions and inspiration. A special thanks goes out to fellow PhD student Maria Helbo Laub-Ekgreen not just for being co-founder of the building 227 social committee but for always listening to me and pulling me back on the horse. I am so glad I got to meet you and really appreciate that I was so fortunate as to share an office with you.

Completing the PhD project period had not been possible without the immense love and support of my family and friends. To my dearest Bjørn thank you for encouraging and always believing in me. Thank you for your endless love through the tough time. Your ability to take my mind of nitrite and make me laugh means the world to me and I would not have made it this far without you.

Sabrine Tauber Pedersen

Kgs. Lyngby, 2018

Abstract

Nitrite is a multifaceted additive contributing to colour and flavour formation as well as extending shelf-life of processed meat products by ensuring oxidative and microbiological stability. It is generally agreed to be a necessary additive especially for its anti-botulinum effect and despite massive research efforts, no true alternative has been found. Application of nitrite in meat curing is however still receiving immense attention for its role in formation of carcinogenic N-nitrosamines. The resulting public scepticism toward nitrite and an industrial desire to lower nitrite addition has created a need for investigations of the existence, formation and functional importance of antioxidative and antimicrobial compounds in nitrite-cured cooked meat products in order to ultimately reduce nitrite addition. Consequently, the focus of this PhD has been on characterising antioxidative and antimicrobial activities in a $\leq 10\text{kDa}$ aqueous fraction of nitrite-cured cooked pork products (NCCPPs) and investigating the impact of processing, including the effects of amount of added curing agents – nitrite and ascorbate.

Three different *in vitro* antioxidant activity assays were applied to $\leq 10\text{kDa}$ aqueous extracts of a selection of nitrite-cured cooked commercial hams (Paper I) as well as model hams (Paper III) and sausages of varying nitrite/ascorbate addition. A clear effect of curing on reducing power and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical scavenging activity were evident for all samples. The results showed these two types of *in vitro* antioxidant activity to be strongly connected with ascorbate, however, whether the correlation was with added or residual ascorbate varied with sample categories. Furthermore, the interplay between added ascorbate and added nitrite seemed to greatly affect the detected *in vitro* antioxidant activity. This could be due to mutual reactions, leading to formation of reaction products of increased or decreased antioxidative properties, compared to the individual reactants. Great attention should also be paid to the added nitrite:ascorbate ratio ($<1:2.3$), in order to avoid conversion to pro-oxidant activities when surpassing an unknown threshold concentration.

A storage experiment comprising the model hams were also conducted (Paper III). Reducing power activity increased with extract concentrations at lower levels of nitrite/ascorbate addition but at higher addition levels reducing power increased with extract concentration, only to decrease once extract concentration had reached a certain level. Interestingly this changed during storage to reducing power activity increasing with extract concentrations for all nitrite/ascorbate addition levels. The same development was shown for residual ascorbic acid. Normalised ABTS radical scavenging activity increased throughout storage, while iron chelating activity tended to increase with storage time in samples of higher nitrite/ascorbate addition. Thus, nitrite/ascorbate addition, beyond a certain threshold concentration, could be affecting a time-depended formation of active iron chelating component(s). No other connections between iron chelation and curing were observed. Overall the results showed that addition of $\leq 150\text{ppm}$ nitrite and $\leq 600\text{ppm}$ ascorbate constituted the tested levels, at which the best overall antioxidative response was obtained.

In the attempt to characterise the very complex ≤ 10 kDa aqueous extracts the samples were subjected to further fractionation using size exclusion chromatography (among others Paper II). It was clear from this characterisation that processing had an impact on chromatographic peaks that coincides with fractions displaying antioxidant activity. In addition to containing residual amounts of additives and active species hereof, the extracts were generally found to constitute a very complex mixture of peptides. Through the characterisation of the extracts, it became clear that the *in vitro* antioxidant activities had to originate from a highly diverse selection of compounds, and that small peptides and certain amino acids e.g. tyrosine, tryptophan, histidine, proline and cysteine may have been of great importance for the *in vitro* antioxidative properties measured in the tested NCCPPs. Other methods including liquid chromatography-mass spectrometry were also employed but it was not possible to obtain a full molecular characterisation of the antioxidative origin. Yet, as part of the extract characterisation the samples were examined for content of S-nitrosated and C-nitrated peptides, yet such peptides were not found. It was speculated that the lack of detectable 3-nitrotyrosine (3NT) might be due to the presence of strong antioxidants or degradation during sample preparation, however, spiking experiments indicated that 3NT might have been degraded by compounds not transferring to the aqueous ≤ 10 kDa fraction during dialysis.

Regardless of differences in product type and processing condition, including type and amount of additives, of the tested NCCPPs no growth inhibitory activities were detected. A potential explanation could be that antimicrobial activities in NCCPPs are in fact related to the curing process, but that the active compounds might somehow be associated with the meat matrix and thus, could not be measured in the aqueous extracts. Alternatively, the tested NCCPP fractions could contain antimicrobial compounds but they were merely tested in too low concentrations to generate a response.

This study has emphasized the importance of the established curing agents – nitrite and ascorbate – for the oxidative stability of cured meat products but has also pointed out, that other compounds such as yet unidentified reaction products of curing agents and meat constituents, as wells as (modified) peptides and amino acids may also contribute to this property. This does, however, need further investigation and a full molecular characterisation for future utilisation in the processed meat industry.

Resumé

Nitrit er et multifunktionel tilsætningsstof, der foruden at bidrage til farve- og smagsdannelse også forlænger holdbarheden af forarbejdede kødprodukter ved at sikre en vis oxidativ og mikrobiel stabilitet. Nitrit er alment anerkendt som et nødvendigt tilsætningsstof i særdeleshed for dets evne til at hæmme *Clostridium botulinum*, og trods megen søgen er der endnu ikke fundet et reelt alternativ. Anvendelse af nitrit til saltning af kød møder dog stadig stor bevågenhed på grund af dets rolle i dannelsen af kræftfremkaldende N-nitrosaminer. Den resulterende forbrugerskepsis mod nitrit og et generelt ønske fra industrien om brug af mindre nitrit, har skabt et behov for undersøgelser af eksistensen, dannelsen samt den funktionelle betydning af antioxidative og antimikrobielle stoffer i varmebehandlede nitritsaltede produkter for med tiden at kunne reducere nitrittilsætningen. Fokus for denne ph.d. har derfor været at karakterisere antioxidative og antimikrobielle aktiviteter i en ≤ 10 kDa vandig fraktion af varmebehandlede nitritsaltede svinekødsprodukter (VNSSP) samt at undersøge effekten af forarbejdning herunder effekten af ændringer i mængden af tilsat nitrit og askorbat.

≤ 10 kDa vandige ekstrakter af et udvalg af kommercielle varmebehandlede nitritsaltede skinker (Paper I) samt model skinker (Paper III) og pølser med varierende nitrit/askorbat tilsætning blev undersøgt med tre forskellige *in vitro* assays til vurdering af antioxidant aktivitet. Der sås en tydelig effekt af nitritsaltningen på reducing power og 2,2'-azino-bis (3-ethylbenzthiazolin-6-sulfonsyre) (ABTS) radikal scavenging aktivitet for alle prøver. Resultaterne viste en tydelig sammenhæng mellem disse to typer af *in vitro* antioxidant aktivitet og askorbat, men hvorvidt den observerede sammenhæng var i forhold til tilsat eller residual askorbat varierede mellem prøvetyperne. Samspillet mellem askorbat og nitrit lod i høj grad til at påvirke den detekterede *in vitro* antioxidant aktivitet. Dette kan skyldes deres indbyrdes reaktioner, som kunne lede til reaktionsprodukter af øget eller mindsket aktivitet sammenlignet med de respektive reaktanter. Man bør være særligt opmærksom på den anvendte nitrit:askorbat ratio ($<1:2,3$), da denne ved overskridelse af en endnu ukendt græseværdi påvirker omslag til prooxidative aktiviteter.

Der blev også udført et lagringsforsøg med model skinkerne (Paper III). I starten af lagringsperioden steg reducing power aktiviteten med ekstraktkoncentrationen for prøver tilsat lavere niveauer af nitrit/askorbat mens den ved højere tilsætningsniveauer steg med ekstraktkoncentrationen, indtil den nåede et vist punkt, hvorefter den faldt igen. Dette ændrede sig dog igennem lagringsperioden til, at reducing power aktiviteten steg med ekstraktkoncentrationen for alle nitrit/askorbat tilsætningsniveauer. Den samme tendens blev observeret for residual askorbat. Normaliseret ABTS radikal scavenging aktivitet steg igennem hele lagringsperioden, mens jern kelerings-aktivitet tendenserede til at stige over lagringsperioden men kun for prøver med højere nitrit/askorbat tilsætningsniveauer. Dette kunne tyde på, at der ved nitrit/askorbat tilsætning over en vis størrelse sker en tidsafhængig dannelse af aktive jern-kelerende stoffer. Der blev ikke observeret yderligere sammenhænge mellem jern-kelering og nitritsaltning. Overordnet viste resultaterne, at

tilsætning af ≤ 150 ppm nitrit og ≤ 600 ppm askorbat udgjorde det testkoncentrationsniveau, hvor det bedste samlede antioxidative respons blev opnået.

I forsøget på at karakterisere de yderst komplekse ≤ 10 kDa vandige ekstrakter, blev prøverne yderligere fraktioneret ved størrelseskromatografi (bl.a. Paper II). Ved denne karakterisering blev det tydeligt at forarbejdning havde en effekt på kromatografiske peaks, der var sammenfaldende med fraktioner, som udviste antioxidant aktivitet. Udover at indeholde små mængder af tilsætningsstoffer og aktive reaktionsprodukter heraf blev det fastslået, at ekstrakterne udgjorde en meget kompleks blanding af peptider. Gennem karakteriseringen af ekstrakterne stod det klart, at *in vitro* antioxidantaktiviteterne måtte stamme fra en samling af meget forskelligartede forbindelser, og at små peptider og visse aminosyrer, f.eks. tyrosin, tryptophan, histidin, prolin og cystein kan have været af stor betydning for de *in vitro* antioxidative egenskaber målt i de testede VNSSP'er. Der blev også brugt andre metoder til karakteriseringen, herunder væskkromatografi-massespektrometri, men det var ikke muligt at opnå en fuld molekylær karakterisering af ophavet af den målte antioxidant aktivitet. Som et led i karakterisering blev prøverne undersøgt for indhold af S-nitrosylerede og C-nitrerede peptider, men sådanne peptider blev ikke detekteret. Det blev overvejet om manglen på 3-nitrotyrosin (3NT) kunne skyldes tilstedeværelse af stærke antioxidanter eller nedbrydning under prøveforberedelsen, men tilsætningsforsøg indikerede at 3NT kan være blevet nedbrudt af stoffer, der ikke er overført til den vandige ≤ 10 kDa fraktion under dialysen.

Uafhængig af forskelle i produkttype og forarbejdning, herunder type og mængde af tilsætningsstoffer, blev der ikke fundet nogle bakterievæksthæmmende aktiviteter. En potentiel forklaring kunne være, at skønt eventuelle antimikrobielle aktiviteter i VNSSP'er er forbundet med nitrit-saltning, så er de aktive komponenter på ukendt vis fysisk associerede med kødmatricen og kunne derfor ikke måles i de vandige ekstrakter. Alternativt var de aktive komponenter blot til stede i for lave koncentrationer til at generere et respons.

Dette studie understreger betydningen af nitrit og askorbat for den oxidative stabilitet af nitrit-saltede kødprodukter men påpeger samtidig, at andre komponenter, så som endnu uidentificerede reaktionsprodukter mellem nitrit/askorbat og kødkomponenter, samt (modificerede) peptider og aminosyrer, også kan have bidraget til denne stabilitet. Yderligere undersøgelser og en fuld molekylær karakterisering er dog nødvendig, før denne nye viden kan tages i brug i industrien.

List of abbreviations

Any abbreviation beyond the commonly used nomenclature e.g. units, amino acids three letter abbreviations and chemical formulas are listed below

3NT	3-nitrotyrosine
ABTS	2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)
Asc	Ascorbate or ascorbic acid (<i>in figures and formulas only</i>)
BCA	Bicinchoninic acid
BPC	Base peak chromatogram
BSA	Bovine serum albumine
CysNO	S-nitrosocysteine (<i>in figures only</i>)
CytC	Cytochrome C
DAD	Diode array detection
dH ₂ O	Deionised water
DMRI	Danish Meat Research Institute
DTU	Technical University of Denmark
E249	Potassium nitrite
E250	Sodium nitrite
E301	Sodium ascorbate
E450, E451	Selection of phosphate textural agents
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EFSA	European food safety authority
ESI	Electrospray ionisation
EU	European Union
FDA	U.S. Food & Drug Administration
GC	Gas chromatography
HENS buffer	Buffer composed of HEPES, EDTA, Neocuproine and SDS
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
IgG	Immunoglobulin G
iodoTMT	Iodoacetyl Tandem Mass Tag™
LC-(ESI)-QTOF	Liquid chromatography- electrospray ionisation-quadrupole time of flight
LC-MS	Liquid chromatography mass spectrometry
LC- MS/MS	Liquid chromatography tandem mass spectrometry
Mb	Myoglobin
MMTS	Methyl methanethiosulfonate
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MWCO	Molecular weight cut-off
NCCPP	Nitrite-cured cooked pork product
PES	Polyethersulfone
PVDF	Polyvinylidene difluoride

QTOF	Quadrupole time of flight
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RP-HPLC	Reverse phase high performance liquid chromatography
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography
SNO-RAC	Resin assisted-capture of SNO-proteins
TBARS	Thiobarbituric acid reactive substances
TBS	Tris buffered saline
TBST-20	Tris buffered saline Tween®-20
UF	Ultrafiltration
VNSSP	Varmebehandlede nitritsaltede svinekødsprodukter (Danish translation of NCCPP)

Sample abbreviations

DH _x	Commercially available nitrite-cured whole-muscle dinner ham (Sweden). The number in the x-position signifies the number of days the ham was stored at 5°C before extraction
SH _x	Commercially available nitrite-cured restructured sandwich ham (Denmark). The number in the x-position signifies the number of days the ham was stored at 5°C before extraction
SPS _x	Commercially available nitrite-cured whole-muscle smoked pork saddle (Sweden). The number in the x-position signifies the number of days the ham was stored at 5°C before extraction
h0/0	Restructured ham produced at DMRI pilot plan with 0ppm added sodium nitrite and 0ppm added sodium ascorbate
h60/240	Restructured ham produced at DMRI pilot plan with 60ppm added sodium nitrite and 240ppm added sodium ascorbate
h150/600	Restructured ham produced at DMRI pilot plan with 150ppm added sodium nitrite and 600ppm added sodium ascorbate
h250/1000	Restructured ham produced at DMRI pilot plan with 250ppm added sodium nitrite and 1000ppm added sodium ascorbate
h500/2000	Restructured ham produced at DMRI pilot plan with 500ppm added sodium nitrite and 2000ppm added sodium ascorbate
h1000/4000	Restructured ham produced at DMRI pilot plan with 1000ppm added sodium nitrite and 4000ppm added sodium ascorbate
s0/0	Wiener sausage produces at DTU with 0ppm added sodium nitrite and 0ppm added ascorbic acid

s0/2000	Wiener sausage produces at DTU with 0ppm added sodium nitrite and 2000ppm added ascorbic acid
s500/0	Wiener sausage produces at DTU with 500ppm added sodium nitrite and 0ppm added ascorbic acid
s500/2000	Wiener sausage produces at DTU with 500ppm added sodium nitrite and 2000ppm added ascorbic acid
s105/240	Wiener sausage produces at DTU with 105ppm added sodium nitrite and 240ppm added ascorbic acid

List of manuscript

Paper I:

Pedersen, S. T., Baron, C. P., De Gobba, C., Duedahl-Olesen, L., Koch, A. G. & Jessen, F. Antioxidative and antimicrobial activity of extracts from nitrite-cured cooked pork products. *Manuscript close to submission*.

Paper II:

Pedersen, S. T., Duedahl-Olesen, L. & Jessen, F. Characterization of low molecular weight peptidic extracts from nitrite-cured cooked pork products. *Submitted to Journal of the Science of Food and Agriculture*.

Paper III:

Pedersen, S. T., Duedahl-Olesen, L., Koch, A. G. & Jessen, F. Effect of storage and varying nitrite and ascorbate concentrations on antioxidative and antimicrobial activity in low molecular weight extracts from nitrite-cured cooked pork products.

Table of Contents

Preface	i
Acknowledgements	ii
Abstract	iii
Resumé	v
List of abbreviations	vii
List of manuscript.....	x
1. Introduction	1
2. Background.....	3
2.1 Nitrite-curing of meat.....	3
2.1.1 The origin of nitrite-curing.....	3
2.1.2 Manufacturing of nitrite-cured meat.....	3
2.1.3 Legislation	4
2.2 Nitrite-induced reactions in meat curing	5
2.2.1 What happens to nitrite once added to meat.....	5
2.2.2 Nitrite and the other curing agents	7
2.2.3 Nitrite and meat components	7
2.3 Oxidation in meat	10
2.3.1 Lipid oxidation and antioxidant.....	11
2.3.2 Protein oxidation and antioxidant.....	12
2.3.3 Curing agents and oxidative stability	13
2.4 Curing and antimicrobial activity	16
2.5 Effects of storage and cooking of cured meat on nitrite and ascorbic acid	17
2.6 Antioxidative and antimicrobial peptides.....	18
3. Materials, methods and methodological consideration.....	21
3.1 Sample material	21
3.1.1 Commercial NCCPPs	21
3.1.2 Model hams	23
3.1.3 Model sausages.....	24
3.2 Methodological considerations.....	25
3.2.1 Removal of potentially disturbing substances	25
3.2.2 Application of in vitro antioxidant assays	25
3.2.3 Selection of microorganism for inhibition studies.....	26

3.2.4 Selection of suitable detection method and sample preparation strategy for 3-nitrotyrosine	26
3.2.5 Developing a chromatographic method for ascorbic acid determination	28
3.3 Methods	30
3.3.1 Basic composition.....	30
3.3.2 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)	31
3.3.3 Biotin switch.....	31
3.3.4 Liquid chromatography-mass spectrometry (LC-MS)	33
4. Results and Discussion	35
4.1 Part A – Commercial NCCPPs	35
4.1.1 Characterisation of antioxidative properties in commercial NCCPPs (Paper I and II)	35
4.2 Part B – Model systems	40
4.2.1 Antioxidant activity in a ham model system (Paper III).....	40
4.2.2 Antioxidant activity in a sausage model system.....	43
4.2.3 SEC on extracts from model hams and sausages and antioxidant capacity of the SEC fractions ..	46
4.3 Part C – Antimicrobial activity.....	54
4.3.1 Studies of bacterial growth inhibition by extracts of commercial and model hams	54
4.4 Part D – Characterisation of constituent components in aqueous $\leq 10\text{kDa}$ NCCPP extracts.....	55
4.4.1 SDS-PAGE and biotin switch.....	55
4.3.2 Mass Spectrometry (MS).....	60
4.5 Part E – Ascorbic acid and free amino acids in colourimetric <i>in vitro</i> assays.....	66
4.5.1 BCA protein assay and ascorbic acid in NCCPPs	66
4.5.2 In vitro reducing power and ABTS radical scavenging antioxidant activity of free amino acids ..	69
5. Summarising discussion and conclusion	77
6. Future work.....	81
7. References	83
8. Appendix	101
Appendix A	103
Appendix B.....	105
Appendix C.....	109

Paper I

Paper II

Paper III

Chapter 1

Introduction

The European processed meat market was valued at \$103.55 billion in 2016 and was predicted a considerable growth during the following years (Market Data Forecast, 2016) and of this market cured meat accounts for the greatest share adding up to 30% (Mordor Intelligence, 2017; Zion Market Research, 2017). The unique Danish lunch tradition of eating rye bread open sandwiches are contributing to a considerably large intake of processed meat in the Danish population. In 2013 processed meat, which includes nitrite-cured meat, was found to constitute almost 30% of the total meat (not including fish) consumed by the Danes (Biltoft-Jensen *et al.*, 2016). Nitrite-curing is a processing technique frequently used in the manufacture of numerous types of processed meat products with the general purpose of extending shelf-life and creating an appealing and microbiologically safe product. In this process nitrite has been pointed out to be a key-responsible for the oxidative and microbial stability of such cured meat products. Despite all the many effective and beneficial attributes of nitrite in regard to food preservation, nitrite is publically perceived as an undesirable and even toxic food additive, the latter mainly referring to correlation with increased risk of certain types of cancer among other caused by the formation of carcinogenic N-nitrosamines (Larsson, Bergkvist, & Wolk, 2006; Larsson & Wolk, 2012; Santarelli, Pierre, & Corpet, 2008). Carcinogenic N-nitrosamines may be formed from nitrite during meat processing but their formation can be limited by lowering nitrite addition (Herrmann, Granby, & Duedahl-Olesen, 2015). Consequently, utilization of nitrites in food is strictly controlled and limited by current EU legislations. Additionally, ever since the EU harmonization concerning food additives in 1995, Denmark has been exempted from following the European legislation concerning nitrite and has set lower limits for its addition to products to be sold in Denmark (EFSA, 2017). In order to retain and preferably to further minimize nitrite addition to meat, it is necessary to acquire further knowledge on the protective behaviour of nitrite and other additives in nitrite-cured products.

The ever growing public scepticism towards artificial food additives has led to an intensification of the search for compounds of natural origin intended to replace unwanted E numbers like nitrite. Focus has to a large extent been on peptides with antioxidative and antimicrobial properties. Such active peptides and also free amino acids have been detected in many foods and food products (de Castro & Sato, 2015), however, it would appear that none or remarkably few investigations have been performed on nitrite-cured cooked meat products. Neither has it been possible to come up with a single non-protein alternative to cover all the properties of the multifaceted nitrite (Sindelar & Milkowski, 2011). Simultaneously, there has been a strong interest from the Danish meat industry to use the lowest possible amount of nitrite and to obtain better knowledge about the metabolism of nitrite in meat products.

The chemical reactions taking place during the nitrite-curing process are still not fully understood, however, it was reported decades ago that nitrite reacts readily with proteins and that only a minor fraction reacts with myoglobin to form the cured meat pigment (Cassens, Greaser, Ito, & Lee, 1979). Thus, a potential strategy for optimizing nitrite addition could be to include investigations of nitrite-induced modification on peptides/amino acids as such modified peptides have previously been found to possess antioxidant properties (Chiueh & Rauhala, 1999; Kanner, 1979; Morrissey & Tichivangana, 1985).

Conclusively, there is a need and an emerging request for investigations of the existence, formation and functional importance of antioxidative and antimicrobial compounds, possibly of protein-origin, occurring during nitrite-curing of cooked meat products in order to ultimately reduce nitrite addition.

With this emerging need constituting the driving force behind the current PhD study the following three working hypotheses were drafted using pork as the selected working matrix:

Hypothesis 1: During the production of cooked pork products with nitrite, antimicrobial and antioxidant compounds are formed.

Hypothesis 2: There are optimal processing conditions for the formation of these antimicrobial and antioxidant compounds.

Hypothesis 3: There are optimal storage conditions to retain the activity of antimicrobial and antioxidant compounds in nitrite-cured pork products.

The work conducted in connection with answering the working hypothesis of this PhD study is documented in the following chapters of this dissertation. After having outlined the motivation and objectives here in Chapter 1, Chapter 2 begins with introducing the concept of nitrite-curing before and now. This is followed by a brief review of the actions and reactions underlying the application of nitrite-curing in meat processing, and finally the chapter finishes with a presentation of antioxidative and antimicrobial peptides. Chapter 3 describes the sample material, any relevant methodological considerations and applied experimental methods not presented in enclosed the papers (Paper I-III). All results obtained during the PhD work are presented and discussed in Chapter 4 and then collectively assessed in a summarising discussion and conclusion in Chapter 5. As a final point, Chapter 6 presents any future work to be conducted.

Chapter 2

Background

The current chapter presents the background information of the PhD study, focusing on the fate and antioxidative and antimicrobial behaviour of nitrite in meat curing. The elucidation of these topics will include an outline of the underlying chemical reaction in order to provide a general overview of present knowledge of nitrite's complex chemistry. Before turning to this, a short introduction to the nitrite-curing process is given.

2.1 Nitrite-curing of meat

2.1.1 The origin of nitrite-curing

Salting of meat is an ancient preservation technique dating back to Antiquity (Binkerd & Kolari, 1975). By binding water, the salt decreases the water activity below levels required for microbial growth and thereby protects meat and fish from spoilage. In the 19th century it became evident that certain salts were more effective in preserving meat. Saltpetre was acknowledged as the activity enhancing salt contaminant which also caused the meat to retain an appealing reddish pink colour (Binkerd & Kolari, 1975; Honikel, 2008). The growing understanding of the chemical role of nitrite in the curing process eventually led industry to fully or partially replacing saltpetre/nitrate with nitrite in curing salts and brines for different technological reasons e.g. shorter curing time, increased production capacity and better control of colour formation and colour uniformity (Binkerd & Kolari, 1975; Honikel, 2008). Nowadays, nitrate is rarely used, except for a few special products requiring a slow curing process, and in products not subjected to heat treatment early after manufacturing (Binkerd & Kolari, 1975; Honikel, 2008; Sebranek & Bacus, 2007).

2.1.2 Manufacturing of nitrite-cured meat

Today meat curing consists of adding salt containing the curing agents nitrite (or nitrate) and ascorbic acid (or ascorbate) and other ingredients such as sugar and spices to fresh meat in order to create a unique flavour and colour. Yet, the primary purpose of nitrite-curing is still to prolong shelf-life by preventing microbiological spoilage, growth of pathogens and oxidative degradation.

Nitrite-curing is used for an immense variety of processed meat products and although a larger number of methods for the addition of the curing salts exist, they are all modifications or combinations of wet-curing and dry-curing. Wet-curing entails pumping, injecting or immersing fresh meat with/into brines consisting of

curing salt dissolved in water before further downstream processing like boiling and smoking. The differences in the processing technologies applied for production of wet-cured (cooked) products, mainly depend on the size of the meat parts used for product manufacture. Regarding larger whole-muscle products an even distribution of the curing brine after injection is ensured by either leaving the product to rest or subjecting it to physical impact e.g. by tumbling (Heinz & Hautzinger, 2007). Afterwards, the meat is prepared for cooking by restricting it to the desired product shape using e.g. string, elastic nets or moulds and is then heated and/or smoked. Wet-cured (cooked) products may also be produced from smaller pieces of meat that are assembled to larger entities. Such reconstituted products may be further subcategorised (Pearson & Gillett, 1996) but for the sake of this thesis the general term restructured products will be used to describe any product made from smaller pieces meat (smaller muscles or muscle parts). After cutting and trimming the meat pieces are injected or if too small tumbled with the curing brine before stuffing into casings or moulds and cooking to an internal temperature of 70°C (72°C) (Heinz & Hautzinger, 2007). Many techniques exist for binding the meat together some of which include addition of gelatine, creating a surface-protein matrix or utilising heat-induced coagulation of liquefied muscle protein released during tumbling (Heinz & Hautzinger, 2007; Pearson & Gillett, 1996). Meanwhile, as the name implies, dry-curing entails adding the curing ingredients without addition of water (Pearson & Gillett, 1996). This is done by rubbing fresh meat with dry-curing salt and then piling the meat e.g. in barrels, vats or on shelves (Heinz & Hautzinger, 2007; Pearson & Gillett, 1996). The curing salts will draw moisture from the meat to create a “brine” which facilitates diffusion of the curing ingredients into the meat (Pearson & Gillett, 1996). Manufacture of some dry-cured products, such as the whole-meat/muscle Spanish *jamón curado* (however, sometimes produced without nitrite/nitrate), will often also include production steps of drying and maturation. The products only subjected to dry-curing are most often intended for consumption in raw state but may be submitted to further downstream processing e.g. smoking.

Slightly overlooked above, products produced from coarsely ground or minced meat may also be nitrite-cured. Within the category of ground or minced meat products, sausages may be perceived as the most important literally comprising hundreds of different products (Pearson & Gillett, 1996). The processing of the individual sausage products are very diverse but generally speaking all ingredients, including curing, taste and textural agents, are mixed to a “batter”, stuffed in casing and subjected to further processing such as heating, smoking, drying or fermentation.

2.1.3 Legislation

Soon after the discovery of nitrite rather than nitrate as the active curing agent, nitrite was integrated in the meat product manufacturing. However, nitrite in itself is a rather toxic compound compared to nitrate and

early misuse ended up having fatal consequences. Consequently, some of the first legal actions regarding the use of nitrite in meat curing were implemented (Honikel, 2008). Today, the use of nitrite and nitrate as food additives are strictly controlled and limited by current EU legislations. The innate toxicity of nitrite, the general notion that nitrite does not remain unchanged in the product during processing (Honikel, 2008), and the later discovery of carcinogenic N-nitrosamines in cured meat, contributes immensely to the establishment of these limits. Whether the limits should be set on added or residual nitrite has been recurrently debated but is set in amended EU legislation as “added amount” due to the nature of the antimicrobial effect of nitrite (Adler-Nissen, Ekgreen, & Risum, 2014; European Union, 2006; Merino, Örnemark, & Toldrá, 2017). This will be further discussed in Chapter 2.4. As already mentioned Denmark are exempted from following the European legislation concerning nitrite and has set lower limits for its addition to products to be sold in Denmark (EFSA, 2017). While EU legislation generally sets limits on the use of nitrite (E249, E250; expressed as sodium nitrite) in meat products at 150mg/kg and in heat-treated processed meat 100–150mg/kg, Denmark sets a general maximum level of only 60mg/kg (European Union, 2008, 2011, 2015). Product specific deviations especially regarding traditional products exist and can be found in Commission Regulation (EU) No 1129/2011 (EU limits - (European Union, 2011)) and Commission Decision (EU) 2015/826 (Danish national limits - (European Union, 2015)).

2.2 Nitrite-induced reactions in meat curing

Processed meat is a highly complex matrix and the many possible oxidation states of nitrogen (Honikel, 2008), and thereby also nitrite, result in a complex web of reactions occurring between the reactive nitrite, meat constituents and other additives during manufacture, processing and storage.

As a consequence of the unique multifaceted function of nitrite in cured meat, in regard to cured colour, cured flavour, flavour protection (as an antioxidant), and as an antimicrobial agent, nitrite has been extensively investigated. However, the ways by which nitrite achieves all these functions are not completely understood within all the above mentioned aspects (Sebranek, 2009). Depending on the conditions of the reaction environment nitrite is by several different mechanisms converted to nitric oxide which is essential for weaving of the reaction web underlying meat curing (Sebranek, 2009).

2.2.1 What happens to nitrite once added to meat

As it was expressed by Cassens *et al.* (1979) not only changes to colour, flavour and shelf-life, but also the fact that added nitrite disappear, is proof that nitrite reacts when added to meat (Cassens *et al.*, 1979). Once nitrite salts are added to meat the nitrite will dissolve in the water phase. In the weakly acidic (pH 5.5-6.0) conditions of meat 99% of the nitrite will exist as an anion (NO_2^-) available to react with H^+ to form nitrous

acid (HNO_2). The small amount of un-dissociated HNO_2 will exist in equilibrium with its anhydride (N_2O_3) which again is in equilibrium with the two oxides; nitric oxide (NO) and nitrogen dioxide (NO_2). The formed NO_2 may react with water forming one molecule of nitric acid (HNO_3 – which may dissociate to nitrate) and one molecule of HNO_2 which re-enters into the nitric oxide reaction cycle. An overview of the reactions described above is presented in Figure 2.1. The NO on the other hand, will be free to react directly or indirectly with a wide variety of compounds such as myoglobin, ascorbic acid, amino acids and other proteins (Honikel, 2008; Skibsted, 2011). Among the subsequent reactions nitrosation (the addition of NO) is of greatest interest to meat curing and the nitrosating agent may be simple and generated in the reactions described above e.g. N_2O_3 or of a more complex nature. Some of these reactions will be touched upon in the following paragraphs.

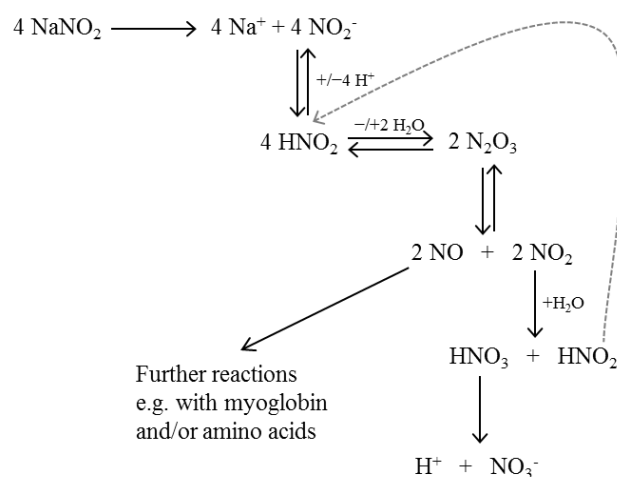


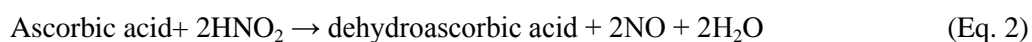
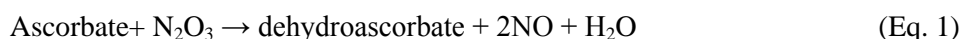
Figure 2.1 Schematic representation of the generation of nitric oxide and other important nitrogen species during curing of meat (modified from (Sebranek, 2009) and (Honikel, 2008)).

Though, use of nitrate salts for curing is rather uncommon today and primarily used for products not intended for heat treatment, curing using a nitrate-source is the foundation for a growing product niche that also include heated products (Sebranek & Bacus, 2007). The so-called “naturally-cured” meat products utilises a nitrate-rich ingredient e.g. celery juice or celery concentrate combined with a starter culture with nitrate reductase activity (Sebranek & Bacus, 2007). The starter culture will cause the nitrate to be reduced to nitrite, which will then react as described above and thereby lead to curing of the meat. Another perhaps more convenient way is to use a nitrate-source reduced beforehand by the ingredient supplier (Sebranek, Jackson-Davis, Myers, & Lavieri, 2012).

Many factors in the complex meat matrix may affect the reactions of nitrite. For instance pH has a great impact on the nitrite reactions during meat curing and as little as a pH decrease of 0.2-0.3 pH units will cause a doubling in the nitric oxide formation rate (Fox, 1974).

2.2.2 Nitrite and the other curing agents

Another very important factor governing nitrite/NO reactions during meat curing are reductants. This includes endogenous reductants such as NADH and sulfhydryl groups, but more important the added ascorbate or ascorbic acid which is added as a so-called cure-accelerator. They are so called for their central role in reactions greatly contributing to the formation of NO. By accelerating the formation of NO the addition of ascorbate/ascorbic acid will consequently lead to a faster turnover of added nitrite and a faster development of cured meat characteristics. Oxidation of ascorbate/ascorbic acid in a redox reaction will yield NO with a 1:2 stoichiometries (Møller & Skibsted, 2002; Skibsted, 2011):



Ascorbate/ascorbic acid may, however, also react with N_2O_3 binding the resulting NO (Skibsted, 2011) which it seems to be capable of passing on to other meat ingredients (trans-nitrosation)(Honikel, 2008). Several (Asc-NO) reaction intermediates have been suggested for any subsequent reactions e.g. for the nitrosylation of myoglobin, but the exact nitrosating agents and reaction pathways (may include thiol groups)(Izumi, Cassens, & Greaser, 1989; Skibsted, 2011) are not fully resolved.

All cured meats have been added sodium chloride in varying amounts (Sebranek, 2009) and according to Sebranek & Fox (1985) the great importance of sodium chloride, as well as sodium nitrite for the distinctive attributes of cured meat, the elimination of either would result in the product no longer being considered as cured. This adds further to the complexity of the chemistry of nitrite in curing of meat as nitrous acid reacts with the chloride ion

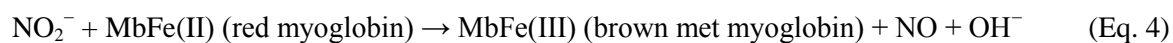


forming nitrosyl chloride (NOCl) which is a stronger nitrosating agent than N_2O_3 (Skibsted, 2011). The difference in reactivity means that formation of NOCl rather than N_2O_3 may have a number of consequences on the curing/nitrosation chemistry, e.g. by affecting reaction rates and changing reaction specificity (Sebranek & Fox, 1985).

2.2.3 Nitrite and meat components

Likely one of the most widely studied compounds related to nitrite-curing is the reaction product of nitrite (in the form of NO) and the meat pigment myoglobin (denoted Mb in the following reaction schemes), nitrosylmyoglobin. Upon addition of nitrite the meat quickly turns brown because the nitrite acts as a strong

heme pigment oxidant while, nitrite itself is reduced and thereby further contributes to the generation of NO (Sebranek, 2009):



By reducing enzymes or chemical reactions with reducing agents like the added ascorbate/ascorbic acid ferric iron is reduced back into ferrous iron (Honikel, 2008). NO may then react with myoglobin (Fe(II)), coordinating to the iron, to form the characteristic cured meat colour, nitrosylmyoglobin (dark red). This is also one of the mechanisms behind the assigning of ascorbate/ascorbic acid as a cure-accelerator (Parthasarathy & Bryan, 2012). Heating of nitrosylmyoglobin-containing meat, like nitrite-cured cooked pork products (NCCPPs), will cause a denaturation of the protein moiety of nitrosylmyoglobin, yet the NO-porphyrin ring system remains. The latter is referred to as nitrosylhemochrome which is heat-stable and responsible for the typical reddish-pink colour of NCCPPs (Honikel, 2008).

Nitric oxide may also bind directly with metmyoglobin (Fe(III)) followed by reduction to nitrosylmyoglobin (Fe(II)) for cured colour formation (Pegg & Shahidi, 1997). Other paths involving metmyoglobin and nitrite or HNO for the formation of nitrosylmyoglobin (Fe(II)) have also been suggested (Fox, 1966; Miranda *et al.*, 2003).

Proteins have a number of potential reaction sites for nitrite (Cassens *et al.*, 1979). As muscles contain a wide spectrum of proteins of different composition and functionality it is not surprising that a considerable share of the nitrite added to meat has been found to be bound to proteins, other than myoglobin (Tricker & Kubacki, 1992).

It has long been established that, of the amino acids constituting meat proteins, cysteine is most likely the most reactive towards nitrite (Olsman & van Leeuwen, 1977). Nitrosation of the cysteine thiol by a nitrosating agent e.g. N_2O_3 (S-nitrosation creating a nitrosothiol (–SNO) side chain) results in the formation of S-nitrosocysteines. However, it appears that S-nitrosation might first occur after nitrosylation of myoglobin (Sullivan & Sebranek, 2012). S-nitrosation of muscle proteins such as myosin has been proven to occur but the extent of occurrence at pH levels equivalent to those found in meat has been disputed (Cassens *et al.*, 1979; Kubberød, Cassens, & Greaser, 1974). The varying levels of detected S-nitrosations in meat are however, likely the result of the formation of –SNO being reversible. Consequently, meat proteins can serve as nitrosating agents (trans-nitrosation) but also a general reservoir for NO and like any residual nitrite this is part of a “NO generating pool” capable of supplying NO for the many reactions taking place during storage and cooking of cured meats – even after ascorbate depletion (Skibsted, 2011). This reversibility of –SNO has also been central to the more recently established importance of –SNO in cell signalling (Miersch & Mutus, 2005).

Another amino acid susceptible to nitrite-induced modifications is tyrosine. Where cysteine was subjected to nitrosation meaning the addition of NO, tyrosine undergoes nitration which means the addition of a nitro (NO_2) group. This occurs on the ortho position of phenolic ring of tyrosine ultimately leading to formation of 3-nitrotyrosine (3NT) (Figure 2.2) (Teixeira, Fernandes, Prudêncio, & Vieira, 2016). Nitration of proteins is a common process taking place at physiological condition, and 3NT has been found to be associated with several diseases e.g. cardiovascular diseases and neurological conditions (Blanchard-Fillion *et al.*, 2006; Mangialasche *et al.*, 2009; Pourfarzam, Movahedian, Sarrafzadegan, Basati, & Samsamshariat, 2013; Sucu *et al.*, 2003). Furthermore, 3NT has been identified in several processed meat systems, isolated myofibrillar proteins and myosin and serum albumin, and has been found to be related to nitrite addition (Feng *et al.*, 2015, 2016; Villaverde, Morcuende, & Estévez, 2014; Woolford, Cassens, Greaser, & Sebranek, 1976).

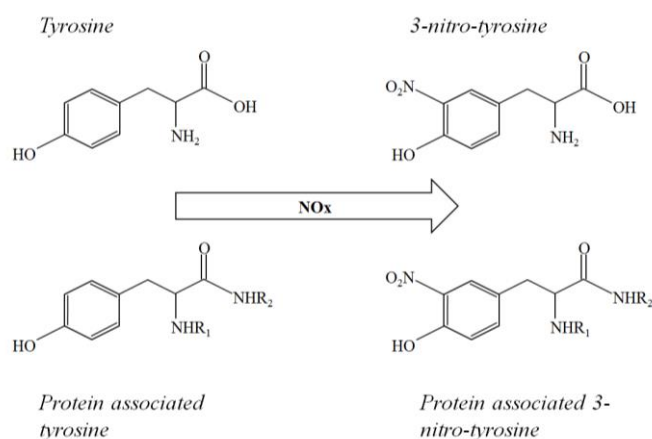


Figure 2.2 Nitration of tyrosine to 3NT (modified from (Ozyurt & Otles, 2017)).

Unlike *in vivo*, the formation of 3NT during food processing and storage is to my knowledge not fully elucidated. Yet, it is likely related to the reactive nitrogen species (RNS) such as peroxynitrite formed during lipid oxidation in cured meat (Villaverde, Parra, & Estévez, 2014) – just as 3NT formation seems to be related to oxidative stress and RNS *in vivo* (Teixeira *et al.*, 2016).

Among the less studied reactions between nitrite and non-heme proteins are the reaction with the peptide bond itself creating nitrosamides (Honikel, 2008). Many other nitrite-induced modification to proteins and amino acids in meat than the once presented here e.g. N-nitrosation of tryptophan (Brown & Stevens, 1975; Ito, Cassens, Greaser, Lee, & Izumi, 1983; Nakai, Cassens, Greaser, & Woolford, 1978) and proline (Dunn & Stich, 1984; Pensabene, Feinberg, Piotrowski, & Fiddler, 1979) on the sidechain nitrogen can occur but is considered outside the scope of this project and will thus not be discussed any further.

Lipids, the other major macronutrient in meat (carbohydrate content is very low), may also react with nitrite. It would appear that nitrite or derivatives hereof may react with the double bonds of unsaturated fatty acids (or its derivatives) e.g. forming alkyl nitrites (Goutefongea, Cassens, & Woolford, 1977; Honikel, 2008). However, the knowledge regarding such reactions is relatively scarce.

The major cause of concern for consumptions of nitrite-cured meat is N-nitrosamines. The main examples of N-nitrosamines found in some heat-treated cured products are N-nitrosodimethylamine and N-nitrosopyrrolidine which are known to be carcinogenic, mutagenic and teratogenic in experimental animals (Pegg & Shahidi, 2004). N-nitrosamines are formed by reaction of nitrosating agents with low molecular weight amines (Parthasarathy & Bryan, 2012), but stable N-nitrosamines are principally formed from N-nitrosation of secondary amines (Honikel, 2008). Prerequisite for N-nitrosamine formation in cured meat products are heating at high temperature ($>130^{\circ}\text{C}$) and naturally also availability of amines and nitrite (Honikel, 2008). As heat-treated nitrite-cured products are made from fresh meat the amount of amines are rather small (Honikel, 2008), and in the case of cooked hams the aimed core temperature is, as mentioned in Chapter 2.1.2, approximately 70°C , thus the N-nitrosamine content of such products may be relatively limited. N-nitrosamines may, however, be present in rubber nettings which may contaminate the edible part of a cooked ham (Fiddler, Pensabene, Gates, & Adam, 1998). It has been established that ascorbate/ascorbic acid is capable of inhibiting N-nitrosamine formation by reacting faster than secondary amines with the nitrosating agent, e.g. N_2O_3 , at the pH relevant for curing of meat (Skibsted, 2011). Basically this would mean reducing N_2O_3 to NO (which is not a nitrosating agent) during ascorbate/ascorbic acid's own oxidation (Parthasarathy & Bryan, 2012). Thus, excess ascorbate added for facilitating curing may also act to prevent potential N-nitrosamine formation and today this ability represents one of the strongest arguments for adding ascorbate/ascorbic acid to cured meat.

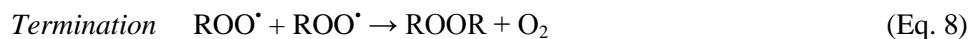
While these are some of the major characterised chemical reactions describing the fate of nitrite during nitrite-curing, there are many other reactions that can and do take place. For example nitrous oxide (N_2O , laughing gas) has been identified in the gases above curing mixtures.

2.3 Oxidation in meat

Oxidative reactions are central to ruining any food. Thus, a basic understanding of the oxidative activities occurring in meat is essential for understanding the shelf-life prolonging effect of nitrite, in regard to oxidative stability of cured meat products. The complexity of meat as a matrix makes it highly complicated to cover all aspects of oxidative activities in meat and thus, it was chosen to focus on oxidation of the two major meat constituents: lipids and proteins.

2.3.1 Lipid oxidation and antioxidant

Oxidation of lipids is the main cause for deterioration of food quality and shelf-life, primarily manifested through formation of objectionable off-flavour and odours – oxidative rancidity. Furthermore, lipid oxidation impairs food quality by prompting loss of essential nutrients and changes in texture and colour, as a consequence of reactions of lipid oxidation products with other food components. Lastly, lipid oxidation may also result in formation of potentially toxic reaction products (Kanner, 1994; Velasco, Dobarganes, & Márquez-Ruiz, 2010). Lipids are oxidised in a complex series of free radical-mediated chain reactions. Lipid oxidation can be divided into three different stages: *initiation*, *propagation* and *termination*:



*"R"= alkyl group of an unsaturated lipid molecule. "H"= α -methylene hydrogen atom easily detachable because of the activating influence of the neighbouring double bond(s).

In the initiation stage, an alkyl radical (R^\bullet) is formed by abstraction of a hydrogen radical from an otherwise stable unsaturated lipid molecule (Eq. 5). In the propagation step, R^\bullet reacts with oxygen to form peroxy radicals (ROO^\bullet) (Eq. 6), which may proceed to react with new lipid molecules giving rise to hydroperoxides (ROOH) as the primary oxidation products and a new R^\bullet that propagate the reaction chain (Eq. 7). Finally, the (generated) radicals react with each other to yield relatively stable non-radical species and thereby terminating the lipid oxidation chain reaction (Eq. 8-10) (Velasco *et al.*, 2010). Hydroperoxides may further decompose into alkoxy (RO^\bullet) and hydroxyl radicals ($^\bullet\text{OH}$) that through different pathways produce a great variety of (volatile) secondary oxidation products (Brady, 2013; Velasco *et al.*, 2010). For reaction (Eq. 5) to proceed requires an initiator and several different circumstances, with different matrix-dependent significance, may lead to initiation of lipid oxidation e.g. light, heat, transition metals, enzymes (lipoxygenase), reactive oxygen species (ROS), metallo-proteins and oxygen accessibility and state (Brady, 2013; Kanner, 1994).

The oxidative stability of foods depends on the balance of the normal oxidation of the substrate and any pro-oxidative and antioxidative reactions and ultimately, which of these reactions are prevailing (Marcuse, 1962). Therefore, a common strategy for controlling lipid oxidation in food is to add antioxidants to supplement any endogenous antioxidants. Antioxidants can be classified based on their mechanism of action as primary/chain breaking antioxidant or secondary/preventive antioxidants. Furthermore, some antioxidants,

e.g. peptides and ascorbic acid, can be referred to as multi-functional as they exhibit both primary and secondary antioxidant properties (Wanasundara & Shahidi, 2005). Primary antioxidants are capable of reacting directly with free radicals converting them to more stable, non-radical products, e.g. by radical scavenging or donating an electron (reducing properties). Thus, primary antioxidant may play a vital role in delaying or inhibiting the initiation step or interrupt the propagation step. Secondary antioxidants exert their antioxidant activity through various mechanisms to slow the rate of oxidation reactions and thus can be said to limit lipid oxidation by indirect mechanisms. These indirect mechanisms include chelating of transition metal ions, singlet oxygen quenching, oxygen scavenging, decomposing hydroperoxide to non-radical species and regenerating primary antioxidant by H donation (Wanasundara & Shahidi, 2005).

2.3.2 Protein oxidation and antioxidant

While the consequences of lipid oxidation for food quality are (almost) immediately perceived by human senses, oxidation of proteins results in more subtle changes with effects on meat texture and juiciness (Lund & Baron, 2010).

Protein oxidation can be induced directly by different ROS, both including free radical and non-radical species such as hydrogen peroxide. Furthermore, radical by-products of other oxidative processes, e.g. ROO[•] from lipid oxidation, can also contribute to induction of protein oxidation (Lund & Baron, 2010; Soladoye, Juárez, Aalhus, Shand, & Estévez, 2015). Generally, oxidation of proteins is believed to proceed through a free radical chain reaction similar to that of lipid oxidation starting with ROS/radical-abstraction of hydrogen from a protein molecule. However, due to the size and complex structure of proteins compared to lipids, free radical-attacks on proteins will result in reaction pathways and reaction products which are much more complex and of much higher variation. All mechanisms of protein oxidation have not been fully established, but the basic chemical mechanisms involved in oxidative modification of proteins are continuously becoming clearer. Detailed descriptions of the known chemical reactions related to protein oxidation will not be giving here but elaborate reviews on the matter has been written Lund, Heinonen, Baron, & Estévez (2011) and Soladoye *et al.* (2015).

Oxidative changes induced to proteins include protein fragmentation through cleavage of peptide bonds, modification of amino acid side chains and formation of covalent intermolecular cross-linked protein derivatives causing mild to severe protein damage. Globular proteins seem to be much more stable towards protein oxidation than the non-globular proteins and of the myofibrillar proteins, myosin appear to be the most susceptible to protein oxidation (Lund & Baron, 2010; Martinaud *et al.*, 1997). The oxidation susceptibility of amino acid also varies, and cysteine, methionine, tyrosine, tryptophan, phenylalanine, histidine, proline, arginine and lysine have been described as particularly prone to oxidation (Lund *et al.*, 2011). Formation of protein carbonyl groups and protein hydroperoxides constitute the most general amino

acid modification, while sulfoxide and sulfone formation from methionine and radical attack on the aromatic ring in tryptophan and phenylalanine constitute examples of the more amino acid-specific oxidative modifications (Lund & Baron, 2010). Protein intra- and inter-molecular cross-linking as a result of protein oxidation are due to formation of disulphide and dityrosine. However, the latter has only been identified in meat model systems and never directly in meat.

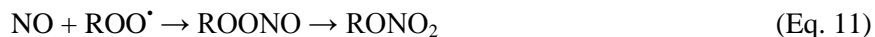
Controlling protein oxidation in meat at the processing end of the food chain is, like for lipid oxidation, achieved by addition of antioxidants. Due to suspected connection between lipid oxidation products and initiation of protein oxidation, inhibition of lipid oxidation is expected to prevent protein oxidation at least to some extent. However, it seems that the classical lipid antioxidants strategies do not inevitably apply to muscle proteins, as research have established that compounds capable of preventing lipid oxidation are not always able to prevent protein oxidation (Lund *et al.*, 2011). In a model system, it was shown that prevention of protein oxidation, using a hydrophilic antioxidant, also had a protective effect on the lipids e.g. the hydrophilic antioxidant Trolox (a vitamin E analogue) prevented oxidation of both protein and lipid fractions. Yet, the lipophilic antioxidants tested were ineffective at preventing protein oxidation (Baron, Berner, Skibsted, & Refsgaard, 2005). In addition to testing the classical antioxidants such as tocopherol, multiple attempts have been made to control protein oxidation by plant phenolics with varying success (see review Lund *et al.* (2011)).

With features such as the nature of the target, location of the site of attack together with the type of the attacking species, having impact on the development of protein oxidation (Dean, Hunt, Grant, Yamamoto, & Niki, 1991; Soladoye *et al.*, 2015) and adding intricacy to the protein oxidation mechanisms (which is also not fully elucidated), makes it difficult to describe the antioxidants kinetics and mechanisms for the inhibition of protein oxidation. Thus, to date no systematic understanding on how proteins can be protected from oxidation by antioxidant exists.

2.3.3 Curing agents and oxidative stability

Nitrite added to meat behaves as an oxidant (Skibsted, 2011). However, immense evidence points to the addition of nitrite to reduce lipid oxidation in meat (Mac Donald, Gray, Kakuda, & Lee, 1980; Willemot, Fillion-Delorme, & Wood, 1987), and several explanations in favour of nitrite as an active antioxidant has been offered. These include iron chelation (Igene, Yamauchi, Pearson, Gray, & Aust, 1985) and stabilisation of unsaturated fatty acid towards oxidation through formation of nitro-nitroso derivatives (Freybler *et al.*, 1993). Oxidation of NO and nitrite, to nitrite and nitrate, respectively, may also, in particular for the former, be perceived as an antioxidant mechanism, as the sequestering of oxygen may contribute to the retardation of lipid oxidation (Honikel, 2008). Yet, it generally seems to be NO that interferes with free radical

intermediates in lipid oxidation. NO reacts rapidly with most radicals including the lipid derived alkyl (R[•]), alkoxy (RO[•]), and peroxy (ROO[•]) radicals, the latter being particularly fast (Carlsen, Møller, & Skibsted, 2005):



This interaction will lead to formation of non-radical addition products, in effect breaking the radical chain processes characteristic of oxidation of unsaturated lipids (Skibsted, 2011). NO may also react rapidly with other radicals implicated in initiating lipid oxidation namely the hydroxyl radical and the superoxide radical anion:



While the interaction of NO with [•]OH (Eq. 12) may alter the course of oxidative reactions in a positive manner, the formation of peroxynitrite (ONOO⁻) (Eq. 13) can have the quite opposite effects. Although, ONOO⁻ may deactivate by isomerisation to nitrate at meat pH, ONOO⁻ is a strong oxidant with the ability to initiate both lipid and protein oxidation. Alternatively it may simply work to deplete the present antioxidant pool (Brannan, Connolly, & Decker, 2001). Oxidative protein modifications induced by ONOO⁻ includes the nitration of tyrosine to 3NT and in that way linking ROS and RNS (oxidative and nitrosative stress).

Ascorbic acid is a well-known multifaceted antioxidant capable of acting as a scavenger of hydrophilic radicals, an oxygen sequencer, and a reducing agent and as a secondary antioxidant, regenerating primary antioxidant such as tocopherol (Mäkinen, Kähkönen, & Hopia, 2001; Niki, 1991). These properties enable added ascorbate/ascorbic acid to have a direct antioxidant effect on lipids in cured meat, e.g. ascorbate may react with oxygen forming dehydroascorbate and thus, reducing the amount of available oxygen for other oxidative reactions (Honikel, 2008). Furthermore, ascorbate/ascorbic acid contribute to the oxidative stability of cured meat through the reactions described above by enhancing the formation of NO (Eq. 1-2). This was clearly demonstrated by Berardo *et al.* (2016) as addition of nitrite, ascorbate or combined addition in increasing order resulted in a decrease in thiobarbituric acid reactive substance (TBARS) values.

It can be said that any antioxidant activity may only be a potential one as most antioxidant may potentially convert to pro-oxidant activities under different circumstances (Marcuse, 1962). Pro-oxidative activities of ascorbate/ascorbic acid involve generation of oxidation initiators/pro-oxidant by reducing Fe³⁺ to Fe²⁺ (Jacobsen, Adler-Nissen, & Meyer, 1999) and by means of Fenton chemistry generating hydroxyl radicals (Skibsted, 2011; Villaverde, Parra, *et al.*, 2014)

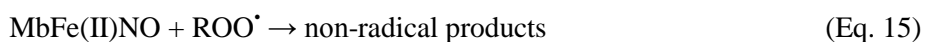
The antioxidant effect of nitrite addition to meat has primarily been directed towards lipid oxidation. Though, there in recent years has been an increase in investigations on the effect of curing agents – nitrite

and ascorbate – on protein oxidation in myofibrillar proteins and different meat products, the conclusions have not been unanimous. Nitrite was both reported to behave as a prooxidant in fermented sausages (Villaverde, Morcuende, *et al.*, 2014) and have negligible or neither pro- nor antioxidative effects in isolated myofibrillar proteins or raw porcine patties (Villaverde, Parra, *et al.*, 2014; Vossen & De Smet, 2015). On the other hand Feng *et al.* (2016) reported finding prooxidative as well as antioxidative effects of adding nitrite to cooked sausages, depending on the amount of added sodium nitrite and what oxidative protein modifications were measured. Berardo *et al.* (2016) also found nitrite to limit protein oxidation on one of the two measured parameters, yet addition of ascorbate in combination with nitrite resulted in increased protein oxidation. Contradictory, a clear antioxidant effect of combined nitrite and ascorbate addition on protein oxidation was found by Villaverde, Morcuende, *et al.* (2014) and Villaverde, Parra, *et al.* (2014). Thus, further clarification of the redox chemistry of nitrite and ascorbate and their interactions with meat proteins, but also the interplay with lipid oxidation, is required to establish the technological importance of nitrite as an antioxidant of protein oxidation in cured meat products.

While myoglobin has been found to be an important initiator of lipid and protein oxidation in meat (Baron & Andersen, 2002; Lund *et al.*, 2011), nitrosylmyoglobin from cured meat has been found to act as a lipid antioxidant (Kanner, Ben-Gera, & Berman, 1980; Morrissey & Tichivangana, 1985; Møller, Sosniecki, & Skibsted, 2002). Decades ago Morrissey & Tichivangana (1985) proposed that this property of nitrosylmyoglobin was related to formation of a stable “myoglobin-NO” complex, formed upon heating of meat, that blocked any catalytic activity of the heme-iron and also prevented the release of the heme-iron, and lastly that nitrosylmyoglobin formed in meat curing acted as an antioxidant *per se*. Regarding the latter, nitrosylmyoglobin(Fe(II)) should be considered as an antioxidant buffer as isotopic labelling studies have indicated that this specie can regenerate active NO derived antioxidants even at low temperature (Andersen, Saaby Johansen, Shek, & Skibsted, 1990). Accordingly, nitrosylmyoglobin(Fe(II)) appears to behave as an antioxidant through dissociation, liberating NO for other antioxidant reactions (Skibsted, 2011):



Additionally, nitrosylmyoglobin (Fe(II)) behaves antioxidatively through direct reaction with free radicals of lipid oxidation such as peroxy radicals (Kanner *et al.*, 1980):



Furthermore, it would appear that the heme centre of myoglobin may also act as an efficient scavenger of the ONOO⁻ oxidant (Carlsen *et al.*, 2005). Although, nitrosylmyoglobin has been found to be important for retaining the oxidative stability of cured meat, the meat pigment itself may also undergo oxidation leading to discolouration and concomitant loss of lipid oxidative stability, as a result of depletion of the

nitrosylmyoglobin (Fe(II)) antioxidant buffer as well as the generation of the oxidation product metmyoglobin (Fe(III)), resulting in prooxidative activities (Skibsted, 2011).

Returning to Morrissey & Tichivangana's (1985) suggestion of the antioxidant activity of the heat stabilised "myoglobin-NO" complex being related to pacifying of the reactive iron, Wettasinghe & Shahidi (1997) demonstrated that a preformed cooked cured-meat pigment (not referred to as nitrosylhemochrome by the authors) acted as an active antioxidant that was more effective than nitrosylmyoglobin at selected concentrations.

As it was described in Chapter 2.2.3 NO will not just associate with myoglobin coordinated to the central iron, but can also bind covalently to amino acids of other proteins with one of these nitrite-induced modifications being of particular interest for the oxidative stability of cured meat. Both S-nitrosoglutathione and S-nitrosocysteine, where the nitrite-induced modification has occurred on a cysteine thiol (hydrogen has been substituted for NO – S-nitrosation), have been found to have antioxidant properties (Chiueh & Rauhala, 1999; Kalyanaraman & Singh, 1998; Kanner, 1979). It is likely, that this effect may be related to S-nitrosocysteine acting as a reducing agent and a nitric oxide donor (Sullivan & Sebranek, 2012), thus contributing to the immense desirable and undesirable consequences of nitrite-curing.

2.4 Curing and antimicrobial activity

As it has already been implied, nitrite is not merely added for colour, flavour and antioxidant purposes, but also as an important antimicrobial agent against both spoilage and pathogenic bacteria, commonly found in meat products. However, the effectiveness of nitrite in controlling or preventing bacterial growth varies between bacterial species, and nitrite has demonstrated markedly stronger antimicrobial properties against Gram positive than Gram negative bacteria (Sindelar & Milkowski, 2012). Nevertheless, Pichner, Hechelmann, Steinrueck, & Gareis ((Abstract) 2006) have reported reduced growth *Escherichia coli* (gram negative) in salami with nitrite compared to a similar uncured product. Though, nitrite has been shown to inhibit many different food pathogens e.g. *Staphylococcus aureus*, *Bacillus cereus*, *E. coli*, *Listeria monocytogenes* and *Salmonella* (See review (Milkowski, Garg, Coughlin, & Bryan, 2010)) most research have been focused on the powerful antimicrobial properties of nitrites towards *Clostridium botulinum* i.e. vegetative cells, spores and toxin production (EFSA, 2003; Sindelar & Milkowski, 2012) due to severity of intoxication by the botulinum toxin.

The specific inhibitory mechanisms of nitrite are not well known. Yet, it is likely that the mechanisms may differ between different bacterial species (Sindelar & Milkowski, 2012) just as the antimicrobial effectiveness differs between species. The marked pH dependence of the antimicrobial effectiveness of nitrite (increased effectiveness with decreasing pH) suggest that the antimicrobial actions of nitrite is

associated with the generation of NO or HNO₂ (Sebranek, 2009) and thus are connected to the nitrite-curing related reaction described in Chapter 2.2 and 2.3. However, confirming the statements above, the bacterial NO tolerance varies, from NO acting as a metabolite for some bacteria to being toxic for others (Møller & Skibsted, 2002). Another direct connection to the curing process is ascorbate/ascorbic acid. Besides taking part in the reactions leading to formation of NO and HNO₂, ascorbate addition has been found, together with nitrite and NaCl, to reduce toxin production by proteolytic *C. botulinum* type A and B (Robinson, Gibson, & Roberts, 1982).

Nitrites exert a concentration-dependent antimicrobial effect in cured meat products but the amount of nitrite needed to inhibit *C. botulinum* can differ from product to product (EFSA, 2003). Yet, both added and residual nitrite has been suggested as important for antimicrobial protection (EFSA, 2003; Sebranek, 2009). EFSA advocates for added nitrite, rather than the residual amount, as contributing to the inhibitory activity against *C. botulinum* and consequently states that “*control of nitrite in cured meat products should be via the input levels rather than the residual amounts*” (EFSA, 2003). The believe that added nitrite rather than residual is providing the inhibitory control during storage (Hustad *et al.*, 1973), suggest the formation of antimicrobial compounds as a result of nitrite-related reactions may be significant (Sindelar & Milkowski, 2012). This could well be nitrosated compounds, as S-nitrosocysteine has been found to act as an anticlostridial agent, though less potent than nitrite, in minced turkey meat (Kanner & Juven, 1980). A general antibacterial relevance of S-nitrosocysteine was further emphasized by Incze, Farkas, Mihályi, & Zukál (1974). Finally, heat treatment by other means than thermal inactivation seem to affect microbiological growth in cured meat as the results by Ashworth, Hargreaves, & Jarvis (1973) points to the formation of an antimicrobial compound in pork when heated with nitrite.

2.5 Effects of storage and cooking of cured meat on nitrite and ascorbic acid

From the proceeding chapters it is very clear that nitrite derived compounds take part in a multitude of reactions in meat curing consequently leading to a decrease in residual nitrite. Residual nitrite levels and thus the generation of new nitrogen species capable of further reactions (Chapter 2.2 and 2.3) in cured meat are affected by many factors. Some of these include originally amount of added nitrite, pH, storage and cooking, meat characteristics, reducing substances, as previously mentioned, and transition metal ions (Lee, Cassens, & Fennema, 1981). Furthermore, some of these factors are interlinked, e.g. that cooking can result in an increase in low molecular weight metals and pH (Barbieri, Bergamaschi, Barbieri, & Franceschini, 2013; Decker, Warner, Richards, & Shahidi, 2005), complicating the general picture.

The greatest loss of nitrite in cured cooked meat products occurs during production to the end of heat treatment at which point nitrite may have been reduced to ≤35% of the originally added amount, and nitrite

levels will continue to drop during any succeeding storage (Honikel, 2008; Hustad *et al.*, 1973). The extent of the decrease in nitrite during storage may depend both on storage temperature and meat pH as Hustad *et al.* (1973) demonstrated nitrite depletion in wiener sausages to occur much slower at 7°C compared to 27°C, while it was reported that higher pH slows down the rate of disappearance of nitrite (see review Honikel (2008))(Nordin, 1969). An unequivocal time point in storage for complete nitrite depletion has not and most likely cannot be determined due to the many influencing factors, however, the decline may well proceed for more than three months (Honikel, 2008; Hustad *et al.*, 1973).

Heat treatment will initiate protein structural changes, e.g. denaturation and aggregation and a cascade of desirable as well as undesirable chemical reactions, e.g. development of desired flavours and lipid and protein oxidation. Yet, meat processing, including heat treatment, in the presences of nitrite, will in addition to the aforementioned yield RNS leading to consumption of nitrite. However, the circumstances for the heat treatment greatly affect the nitrite turnover. The decreasing effect of increasing pH on nitrite depletion also applies during heat treatment (see review Honikel (2008)). Furthermore, heating at higher temperatures will, on a general basis, result in a greater loss of nitrite (Honikel, 2008). However, in a study of nitrite reaction kinetics by Barbieri *et al.* (2013), smaller changes in heating temperature combined with changes in added nitrite did not result in an equally simple residual nitrite/temperature correlation, but the rate of nitrite disappearance was higher at test temperatures of 65°C or above. As previously mentioned the presences of the reducing ascorbate/ascorbic acid will result in a faster turnover of added nitrite, however, heating will slow down this effect (Honikel, 2008). This is accentuated even further by the addition of polyphosphates (Gibson, Roberts, & Robinson, 1984). Finally it should be noted that ascorbate and ascorbic acid are rather labile compounds, thus both storage and processing may lead to significant losses (Decker *et al.*, 2005; Klimczak & Gliszczyńska-Świąło, 2015).

2.6 Antioxidative and antimicrobial peptides

The biological tissue originally constituting any food contains several antioxidant systems to maintain an optimal antioxidant/pro-oxidant balance, which includes free radical scavenger (e.g. ascorbate and tocopherol), metal chelators (e.g. organic acids) and enzymes inactivating ROS (e.g. catalase and superoxide dismutase) but also, non-enzymatic proteins contribute to the antioxidative reactions (Elias, Kellerby, & Decker, 2008). However, these systems may be disturbed when the tissue is processed into food as different processing steps may result in introduction of oxygen, removal and destruction of natural endogenous antioxidants and increasing pro-oxidative factors (Elias *et al.*, 2008). Thus, it is often necessary to consider addition of antioxidant compounds, such as nitrite and ascorbate in the case of processed meat products. However, proteins and more so peptides have great potential as antioxidant additives in food as they can inhibit oxidative processes through multiple pathways such as free radical scavenging, metal chelating,

acting as reducing agent and altering of the physical properties of a food system (Elias *et al.*, 2008). Consequently, there has, through the past few decades, been a massive interest in antioxidant (and antimicrobial) peptides, among others, to find natural substitutes for the strictly regulated and potentially health hazardous classic synthetic food additives. As for any other antioxidant, for a protein to be a good antioxidant, in particular in regard to radical scavenging, it needs to be more oxidatively labile than unsaturated fatty acids, and the resulting protein radical should be stable enough not to induce any further oxidation.

Antioxidant peptides are generally short peptides that may be hidden and inactive in the sequence of a parent protein but can be released and/or activated during food processing, by hydrolysis using commercial enzymes or lastly during gastrointestinal digestion. It is approaching general perception that peptides have considerably better antioxidant activities than their parent proteins and constituent amino acids (Elias *et al.*, 2008), yet the sequence and presence of certain amino acids are of great importance (Chen, Muramoto, Yamauchi, & Nokihara, 1996). Histidine, tyrosine, tryptophan, methionine, cysteine, phenylalanine and proline are some of the amino acids most frequently listed to enhance antioxidant activity when being part of the peptide sequence (Di Bernardini *et al.*, 2011), but also the presence of some of the remaining hydrophobic amino acids are thought to enhance peptide antioxidant activities (Ren *et al.*, 2008). However, studies have also shown that certain amino acids may also be antioxidative on their own (Marcuse, 1962).

So far antioxidant peptides have been detected in many different food and food related matrixes including matrixes of animal origin. This includes extracts and protein hydrolysates of porcine proteins/tissues (Damgaard, Lametsch, & Otte, 2015; Saiga, Tanabe, & Nishimura, 2003), bovine tissues (Di Bernardini *et al.*, 2011), poultry tissues (Sacchetti, Mattia, Pittia, & Martino, 2008) and fish tissue (Farvin *et al.*, 2014) but also from dry-cured hams and sausages which have undergone processing intended protein breakdown (Mora, Escudero, Fraser, Aristoy, & Toldrá, 2014; Sun *et al.*, 2009; Zhu *et al.*, 2013). However, to my knowledge, the number of known antioxidant peptides extracted directly from untreated animal tissue is rather limited and includes the two histidyl dipeptides carnosine (β -alanyl-L-histidine) and anserine (N- β -alanyl-1-methyl-L-histidine) (Gil-Agustí, Esteve-Romero, & Carda-Broch, 2008) and glutathione (de Castro & Sato, 2015).

Antimicrobial peptides are an essential part of the innate immune system and as such constitute an organism's first line of defense against colonization by exogenous microorganisms (Zhang, Ross, & Blecha, 2000). Antimicrobial peptides are widely distributed in nature and more than 800 antimicrobial peptides have been described in plants and animals (Boman, 2003). These peptides are generally short (<50 amino acids and <10kDa) and composed primarily of cationic and hydrophobic amino acids, which are accounting for up to 50% of the amino acid composition (Najafian & Babji, 2012). These features are central for one of the primary antibacterial mechanisms of creating channels in the microbial cell membrane increasing

permeability (Udenigwe & Howard, 2013). In addition to being naturally “omnipresent”, peptides with antimicrobial activity have also been identified in several protein hydrolysates (Review: (de Castro & Sato, 2015)) and in dry-cured meat products (Castellano *et al.*, 2016) undergoing extensive protein breakdown during maturation and/or fermenting steps in the processing procedure. However, to my knowledge the information on meat-protein related antimicrobial peptides, not submitted to intended protein hydrolysis, are very limited. Furthermore, the research within food-derived antimicrobial peptides has mainly been oriented towards their possible application in medicine and functional foods, rather than their potential as food-grade bio-preservatives and natural role in food shelf-life.

Chapter 3

Materials, methods and methodological consideration

The experimental work constituting the foundation for the current PhD study consisted of a broad variety of methods ranging from advanced chromatographic and mass spectrometric methods to less apparatus demanding methods e.g. dry matter determination. The majority of the specific methods employed during the studies are described in the enclosed Papers. In consequence this chapter aims to present any remaining information essential for the research results. Firstly, this includes a presentation of the sample material which has been analysed. Secondly, a short discussion of the methodological consideration in regard to the selection and development of experimental methods and thirdly, a brief description of any essential methods not found in the Papers.

3.1 Sample material

The processed meat market is dominated by poultry but pork are second in line, and in Europe, consumption of pork is reported to be the largest at 32.3 kg per capita (Mordor Intelligence, 2017; Zion Market Research, 2017) greatly contributed to by Denmark exporting pork meat for approximately 30 billion DKK in 2016 (Landbrug & Fødevarer, 2017). In Northern Europe nitrite-curing is mostly done in combination with heating and/or smoking, and in Denmark the wet-curing techniques are distinctly the most commonly used. Thus, it was decided to focus the investigations on wet-cured NCCPPs.

3.1.1 Commercial NCCPPs

It was decided to initiate the investigations of antioxidant and antimicrobial activity in NCCPPs on a selection of commercial products. As much of the research in the field of antioxidant and antimicrobial properties performed on non-dry-cured pork has been conducted in minced or comminute products, it was decided to initially focus the investigations on products made up of larger pieces of meat such as hams.

A set of three different types NCCPPs were kindly donated to Danish Meat Research Institute (DMRI) from undisclosed collaboration partners. The sample material comprised a sandwich ham (SH) from Denmark and a boiled dinner ham (DH) as well as a smoked pork saddle (SPS) from Sweden. Pictures of the three products can be found in Figure 3.1 while Table 3.1 contains product specifications.

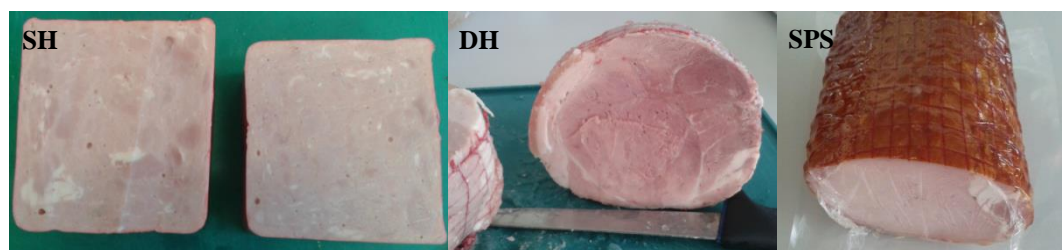


Figure 3.1 The three commercial NCCPPs investigated. SH: cross-section of the restructured boiled sandwich ham, DH: cross-section of boiled dinner ham and, SPS: surface and cross-section of boiled and smoked pork saddle.

Table 3.1 Product specifications for the three commercial NCCPPs either obtained directly from manufacture or stated on the label

	pH	Added preservative	Added amount of preservative	Added antioxidant	Added textural agents
Sandwich ham (SH)	6.0-6.1	nitrite*	60ppm	E301	-
Dinner ham (DM)	6.09	E250	120ppm	E301	-
Smoked pork saddle (SPS)	6.17	E250	120ppm	E301	E450, E451

*The form of the added nitrite is unknown

The sandwich ham was a ready-to-eat luncheon meat product that belonged to the category of restructured meat products where smaller pieces of meat were subjected to curing brine prior to boiling stuffed into a plastic casing. The remaining two products were whole-muscle products most likely injected with curing brine prior to boiling and smoking (only the pork saddle is smoked). The exact manufacturing procedure of all three products was undisclosed by the producers. The sandwich ham was delivered from the manufacturer in bulk while the whole-muscle products, DH and SPS, were supplied from the manufacturer in consumer packaging intended for the Swedish market. A basic compositional analysis of the three hams was conducted and the result is presented in Table 3.2.

Prior to sampling for the individual analytical methods a larger piece of the ham, of approximately 100g, was minced to ensure a homogenous sample. The remaining skin and the excess subcutaneous fat on DH (Figure 3.1) were trimmed before mincing as it was decided to focus the investigations on meat alone (except for fat marbling). If any antioxidant or antimicrobial compounds with importance for the shelf-life of the entire product were present predominantly in the fat and skin of DH these would have been lost. The smoke-coloured surface of the pork saddle (Figure 3.1) was not removed but was considered a part of the meat. Furthermore, the smoke-coloured surface was expected to be consumed by the majority of Scandinavian consumers, unlike the fat and skin on the dinner ham.

Table 3.2 Content of fat, protein, dry matter, ash, salt and residual nitrite and ascorbic acid in the meat of the three tested commercial NCCPPs (mean \pm standard deviation, n=2)

	Fat (%)	Protein (%)	Dry matter (%)	Ash (%)	Salt (%)	Residual Nitrite (ppm)	Residual ascorbic acid (ppm)
Sandwich ham (SH)	3.6 \pm 0.1 ^a	17.2 \pm 0.2 ^a	25.2 \pm 0.3 ^a	4.0 \pm 0.007 ^a	2.9 \pm 0.2 ^a	7.3 \pm 0.1 ^a	354.0 \pm 9.5 ^{a‡}
Dinner ham (DH)	11.1 \pm 0.8 ^b	22.4 \pm 0.1 ^b	35.5 \pm 0.5 ^b	3.8 \pm 0.002 ^b	3.2 \pm 0.3 ^a	4.0 \pm 0.03 ^b	100.9 \pm 8.2 ^{b‡}
Smoked pork saddle (SPS)	9.6 \pm 0.2 ^b	18.7 \pm 0.3 ^c	30.4 \pm 1.8 ^{ab}	4.9 \pm 0.06 ^c	3.6 \pm 0.1 ^a	10.7 \pm 0.3 ^c	445.6 \pm 18.7 ^{c‡}

* Values in the same column follow by different letters are significantly different (p<0.05). Bonferroni multiple testing corrections are included on dry matter and ash.

‡n=6.

3.1.2 Model hams

In order to evaluate the impact of nitrite and ascorbate alone, the NCCPP matrix needed to be uniform and thus a set of six model hams were kindly manufactured at DMRI's pilot plant according to our wishes. As nitrite and ascorbate are usually added in combination and by rule of thumb in a 1:4-ratio, the hams were produced with parallel and continuously increasing levels of the two curing agents. The levels of addition were chosen based on EU and Danish legislation. Furthermore, a couple of extremes were included to ensure a visible antioxidative and antimicrobial effect for further analysis, if the effect in the real life resemblance samples would be too small for detection by the selected methods. Lastly a reference with no added curing agents was also included. The final concentrations were 0, 60, 150, 250, 500 and 1000ppm of added sodium nitrite and 4 times as much sodium ascorbate. The individual model hams will be referred to as added sodium nitrite slash added sodium ascorbate and included an "h" prefix (h for ham) e.g. h60/240.

The model hams belonged to the category of restructured meat products like the commercial SH. The main recipe for the model hams constituted pork meat (75%), water (20%), salt (2.1%), skin protein (*Danish: sværprotein*), phosphate, potato starch and dextrose with a final water content of approximately 75%. The main recipe was identical for all hams only changing the added amount of sodium nitrite and ascorbate. The basic composition of h0/0 is presented in Table 3.3 and was only significantly different from SH in regard to dry matter.

Table 3.3 Content of fat, protein, dry matter, ash, salt and residual nitrite and ascorbic acid in the model ham without added curing agents (mean \pm standard deviation, n=2)

	Fat (%)	Protein (%)	Dry matter (%)	Ash (%)	Salt (%)
h0/0	1.8 \pm 0.2	17.4 \pm 0.1	23.9 \pm 0.2 [†]	3.2 \pm 0.03 [†]	2.5 \pm 0.05 [†]

[†]n=3

A larger piece of the hams, of approximately 100g, was minced prior to sampling for the individual analytical methods to ensure a homogenous sample.

3.1.3 Model sausages

In addition to evaluating the impact of parallel and continuously increasing levels of nitrite/ascorbate (model hams) a small study was set up to evaluate the impact of shifted nitrite and ascorbate addition. This was supposed to give an impression of the importance of any potential interactions between nitrite and ascorbate for detection of *in vitro* antioxidant activity. To accommodate the challenges of the small batch setup and ensuring an even distribution of the ingredients a minced product in the form of wiener sausages were selected as the NCCPP sample matrix. The sausage meat was prepared from minced pork meat with 25% fat from the local butcher (67%), tap water (26.5%), potato flour (4.5%) and sodium chloride (2%). The thoroughly mixed sausage meat was split into smaller portions and each added the appropriate amount of ascorbic acid and sodium nitrite according to the matrix design presented in Figure 3.2.

NO ₂ ⁻ (ppm) \ Asc (ppm)	0	60	105	150	500
0					
50					
240					
600					
2000					

Figure 3.2 Composition of sample material for the sausage experiment with shifting levels of sodium nitrite (NO₂⁻) and ascorbic acid (Asc) addition to wiener sausages. All green combinations were produced.

The sausages were stuffed in sheep casings and heated for 50min at 70°C. Like for the model hams the model sausages will be referred to by added sodium nitrite slash added ascorbic acid and included an “s” prefix (s for sausage).

As for the hams larger amounts of the sausages, approximately 100g, were also minced prior to sampling to ensure a homogenous sample. The sheep casing was removed before mincing.

3.2 Methodological considerations

3.2.1 Removal of potentially disturbing substances

Meat constitutes a highly complex matrix. In addition to the immense quantity of macro- and micro molecules originating from the pig, processed pork meat also contains additives and reaction products of these. Thus, when producing the aqueous ≤ 10 kDa extracts constituting the foundation of the PhD study, the extract may be expected to be equally as diverse containing many low molecular weight compounds that might induce an antioxidative or antimicrobial responds. In the attempt to focus the search for antioxidative or antimicrobial compounds on peptides and other larger molecules it would be preferable to rid the samples of interfering compounds e.g. salts and acids. Especially ascorbic acids added during curing may overshadow the antioxidant properties of other active compounds present/generated in NCCPPs. Different clean up strategies e.g. dialysis against deionised water (dH₂O) was tested without a satisfying result. This will require more attention in case of further investigations.

3.2.2 Application of *in vitro* antioxidant assays

Applying *in vitro* colourimetric assays for the detection of antioxidant properties is a highly convenient and fast approach. Furthermore, it constitutes a great tool for screening of samples for antioxidant properties. However, the effectiveness of antioxidants in food not only depends on their chemical reactivity (e.g. radical scavenging and metal chelation), but also on their physical location, interaction with other food components (e.g. lipid partitioning and the presence of pro-oxidants) and environmental conditions such as pH. Thus, it must be kept in mind that using *in vitro* assays can only provide an indication of sample components' ability to and importance for inhibiting oxidative degradation in this case in meat. Hence, in order to give further indication of whether a detected *in vitro* antioxidant activity, contribute to a slower oxidative deterioration in nitrite-cured meat, it would be necessary in due time to test the antioxidative properties in a representative model system and measure oxidation products (e.g. primary and secondary lipid oxidation products, free thiol groups).

3.2.3 Selection of microorganism for inhibition studies

As previously mentioned, the majority of research on the antimicrobial effect of nitrite has been aimed towards *C. botulinum* i.e. vegetative cells, spores and toxin production, due to the severity of the consequences of botulinum toxin production (King, Glass, Milkowski, Seman, & Sindelar, 2016). Although, antimicrobial properties of nitrite have also been investigated against other food pathogens the research is limited in comparison. *L. monocytogenes* is a genuine concern to manufactures of ready-to-eat meat products. The ubiquitous nature and unique capacity to grow at refrigeration temperatures makes *L. monocytogenes* a highly problematic contaminant of the post-thermal-processing environment that in the case of product contamination may continue to grow during refrigerated storage. Although, the occurrence of listeriosis is relatively rare compared to other foodborne illness e.g. salmonellosis, it may become severe for people with lowered immune defence and the mortality rate among patients with severe symptoms is as high as approximately 25%. In pregnant women listeriosis can furthermore result in spontaneous abortion (Fødevarestyrelsen, 2017). As mentioned above, infection with *Salmonella* is far more common than infection with *Listeria*, with 88,715 and 2,161 confirmed cases, respectively, in EU in 2014. Salmonellosis was the second most common human zoonosis in EU in 2014 only surpassed by campylobacteriosis (236,851 confirmed cases) (EFSA, 2015b). Furthermore, it was estimated that pork meat (Danish produced and imported) was in the top three of most important food sources of salmonellosis in Denmark in 2016 and that the serovar *S. Typhimurium* is the most common (Helwich, Christensen, & Müller, 2016). For these reasons it was decided to test the aqueous $\leq 10\text{kDa}$ extracts for antimicrobial activity against *L. monocytogenes* and *S. Typhimurium*.

3.2.4 Selection of suitable detection method and sample preparation strategy for 3-nitrotyrosine

Due to the unique absorbance spectrum of 3NT (Figure 3.3) quantification is possible by a simple spectrophotometrical measurement at approximately 430nm under alkaline conditions (Crow & Beckman, 1995). This approach was applied successfully in the recently published work by Vossen & De Smet (2015) and Feng *et al.* (2016), and we were also able to detect alkaline solution of 3NT using the available equipment at the National Food Institute, Technical University of Denmark. The simplicity of this detection method was very appealing; however, the method requires relatively large amounts of relatively pure nitrated proteins (thus TCA precipitation was applied by Vossen & De Smet (2015) and Feng *et al.* (2016)). Furthermore, it is easily disturbed by compounds with an intrinsic absorbance in the 400-500nm range (Crow & Beckman, 1995). Attempting to measure absorbance on an aqueous $\leq 10\text{kDa}$ extract of h500/2000 without any additional clean up gave no remarkable responds. Thus, another potentially more advanced method was considered for providing evidence for the presence of 3NT in NCCPPs.

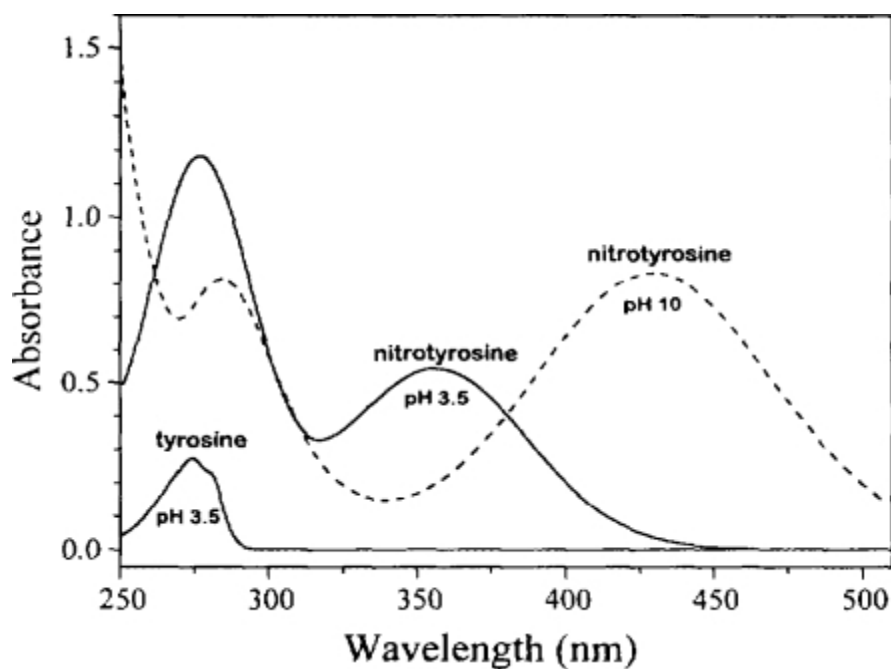


Figure 3.3 UV absorbance spectra of 0.2mM 3NT (nitrotyrosine) at pH 3.5 and pH 10 as well as 0.2mM tyrosine at pH 3.5 (Crow & Beckman, 1995).

A variety of methods for the detection of 3NT have been employed including immunoassays, high performance liquid chromatography (HPLC) methods with different detection methods as well as gas or liquid chromatography (GC or LC) coupled to single or tandem mass spectrometry (MS or MS/MS) (Review: Duncan (2003) and Teixeira *et al.* (2016)). The listed methods vary greatly in complexity, sensitivity, selectivity, availability and costs. Thus, a suitable compromise should be considered when choosing the best fit for the purpose. The use of MS offers a robust and unambiguous compound identification and where GC requires extensive sample preparation involving derivatisation for improvement of volatility, LC-MS and LC-MS/MS provides greater flexibility. Therefore, it was decided that utilising LC-MS/(MS) for the detection of 3NT would be best suited for the current purpose. The selection, testing and optimization of the LC-MS set up were done by collaboration partners at University of Copenhagen, Department of Food Science.

It is a common approach to subject protein containing samples to peptide bond cleavage prior to 3NT detection. It was decided to follow this common practice as this would enable targeting of the investigation to a single compound and thus reduce the complexity of the data treatment. Such a cleavage may be achieved by either acidic or alkaline hydrolysis or enzymatic digestion, yet all of which may cause artefact formation or loss of 3NT (Duncan, 2003; Yang, Zhang, & Pöschl, 2010). Enzymatic digestion was rejected as proteolysis is seldom complete and furthermore autolysis of the protease may disturb the results (Crow & Beckman, 1995). Tyrosine has been reported to undergo nitration under acidic conditions in the presence of only minute amounts nitrite (Shigenaga *et al.*, 1997). As we expected the NCCPP samples to contain residual

nitrite alkaline hydrolysis was tested in the hope of avoiding this potential artefact formation. However, the procedure gave rise to a cloudy appearance of the hydrolysed aqueous ≤ 10 kDa extract samples and nothing was detected by the optimised LC-MS method. The reports on advances/applicability of acid hydrolysis are contradictory (Crow & Beckman, 1995; Delatour *et al.*, 2007). However, addition of phenol have been found to reduce (but not eliminate) nitration of tyrosine by acting as a radical scavenger (Shigenaga *et al.*, 1997). However, based on results from initial investigations and for toxicological reasons it was decided to carry out the main experiments with a simple HCl hydrolysis without addition of phenol.

3.2.5 Developing a chromatographic method for ascorbic acid determination

After futile testing of an enzyme-based commercial kit for colourimetric determination of L-ascorbic acid in foodstuff (Cat no. 10 409 677 035, r-biopharm, Roche diagnostics, Basel, Switzerland) it was decided to develop a chromatographic method for the detection of ascorbic acid. The following summarizes the most important points from the process of developing and validating an HPLC method for ascorbic acid detection.

Ascorbic acid exhibit higher stability at low pH and generally a pH in the proximity of 2.1 has proved useful in stabilising ascorbic acid during sample preparation (Nováková, Solich, & Solichová, 2008). For this reason most analytical methods include extraction under acidic conditions. There is a general tendency towards using meta-phosphoric acid (occasionally in combination ethylenediaminetetraacetic acid (EDTA) or oxalic acid) for the extraction of ascorbic acid from food matrixes, including meat (Kall & Andersen, 1999; Klimczak & Gliszczyńska-Świgło, 2015; Kutnink & Omaye, 1987; Valls, Sancho, Fernández-Muiño, Alonso-Torre, & Checa, 2002). Thus, the sample preparation method described Valls *et al.* (2002) used on commercial cooked sausages was adopted with some slight modification (see method description in Paper I & III). Extra attention was paid to protecting the samples from light as ascorbic acid has been proved to be very susceptible to degradation as a consequence of exposure to light (Iwase, 2000).

Reverse phase (RP) chromatography is widely used for the determination of ascorbic acid, yet these methods frequently suffer from poor resolution (Nováková *et al.*, 2008). To circumvent this issue requires eluent(s) of high water percentage combined with low pH. However, it is well known that aqueous mobile phases not containing an organic modifier can negatively influence separation efficiency on a C18 stationary phase and may even cause a collapse of stationary phase with long-term use (Przybyciel & Majors, 2002). Thus, it was chosen to use water of pH=3 with 5% methanol in an isocratic elution. This was however during further optimization changed for gradient elution including a period of 100% methanol post-analyte elution for cleaning of the column.

Ascorbic acid absorbs UV light with the absorption maximum in the range 244-265nm depending on the composition of the mobile phase. Consequently, it was chosen to utilise UV detection (diode array detection

- DAD) at 254.4nm which corresponded to the median of the presented wavelength range and it is furthermore the most frequently applied when consulting published methods.

As erythorbic acid is occasionally added to cured meat products instead of ascorbic acid, the method was also tested for the detection of the stereoisomer. The method was capable of selectively distinguishing between ascorbic acid and erythorbic acid (Figure 3.4A). The poorly separated cluster of three peaks around 5min was identified to be related to dehydroascorbic acid. Some slight drifting was experienced in the retention time between sample runs and injections. This can also be seen from the sample chromatogram in Figure 3.4B.

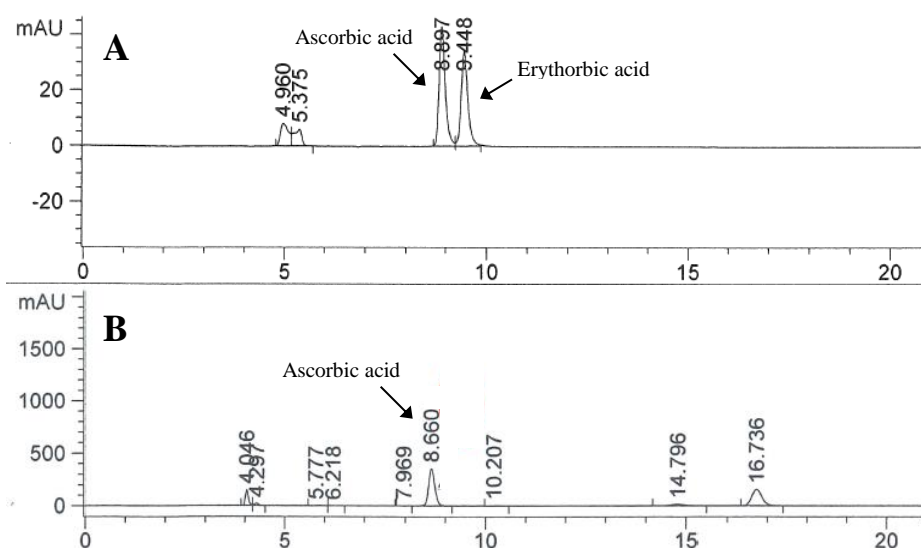


Figure 3.4 HPLC chromatograms from A: the injection of a mixed standard of ascorbic acid and erythorbic acid (5µg/mL) and B: the injection of an ascorbic acid-extract of the NCCPP used for method development.

The validity of the ascorbic acid detection method was tested in multiple ways. Linearity (only for ascorbic acid) was investigated by means of external standards calibration curves of ascorbic acid separately or combined with erythorbic acid in the range 0.5-30µg/mL in 6-8 concentration levels. Running 4 external calibration curves on separate occasions (≥ 4 monthly intervals) and running each calibration curve twice, more than 5 hours apart, resulted in slopes of 70.2 ± 1.3 , $R^2 = 0.998 \pm 0.001$. An example of an external standard calibration curves are presented in Figure 3.5.

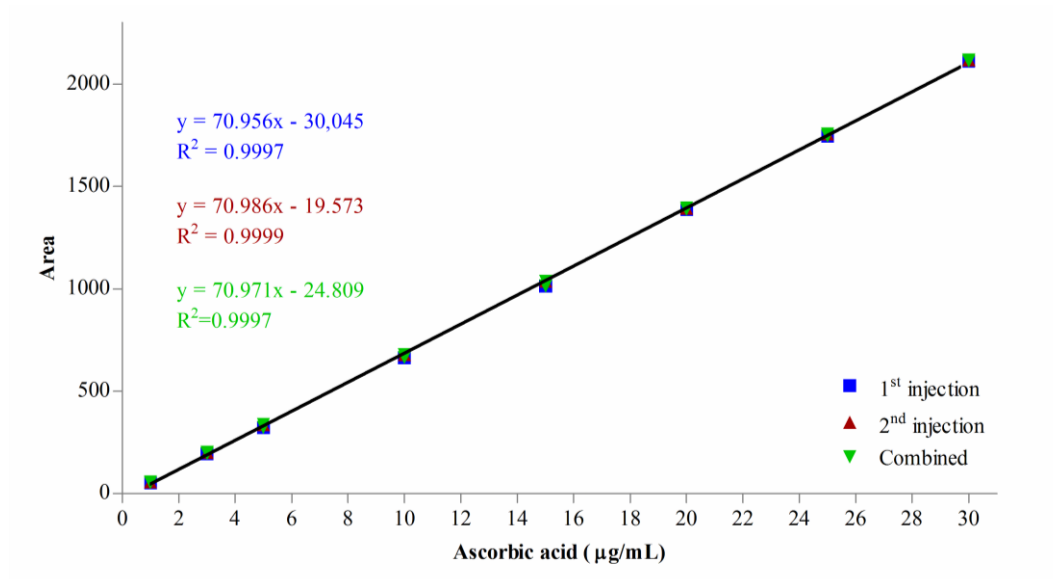


Figure 3.5 Ascorbic acid calibration curve for the two injections of the same calibration curve run approximately 10 hours apart.

The complete analysis on identical NCCPP samples was conducted on several different days and several times on the same day in order to determine inter- and intraday repeatability. The relative standard deviation (not in full compliance regarding number of replicates) on inter- and intraday determinations were 7.8% and 6.2% respectively. Finally the degree of recovery was determined by spiking a NCCPP sample with three different concentration of a standard solution on two different days and was found to be $100.1 \pm 3.1\%$.

3.3 Methods

3.3.1 Basic composition

The lipid content in the NCCPPs were analysed according to a modified Bligh and Dyer extraction method (Bligh & Dyer, 1959) utilizing a reduced amount of chloroform and methanol.

The method builds on the principle of extracting all lipids from the sample (approximately 10g of minced sample) into the methanol/chloroform phase and weighing the oil left after full evaporation of the solvent from a known amount of the Bligh and Dyer extract.

Protein was determined as Kjeldahl nitrogen according to AOAC Official Method. 39.1.19 981.10 (AOAC Official Methods of Analysis, 1996). Kjeldahl determines the content of organic as well as some inorganic nitrogen after a full destruction and conversion to ammonia which is quantified by titration. The protein content was then determined using a conversion factor of 6.25.

The dry matter content was determined by evaporation of all water at 105°C for 24 hours. The dry matter content is expressed in percent and calculated according to

$$\%Dry\ matter = \frac{mass\ of\ dry\ sample \cdot 100\%}{mass\ of\ wet\ sample} \quad (Eq. 16)$$

The ash content was measured by burning the dried sample at 600°C for approximately 24 hours. The temperature was set at 100°C for the first hour and afterwards increased to 600°C. The ash content is reported as

$$\%Ash = \frac{mass\ of\ ash \cdot 100\%}{mass\ of\ wet\ sample} \quad (Eq. 17)$$

3.3.2 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Separation of the extract components prior to the intended immunoblotting for detection of S-nitrosated peptides were conducted using the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) technique. Several minor modifications were implemented, such as using different sample buffers (e.g. commercial buffers, laemmli buffer and urea buffer) as well as different gels and corresponding running buffers (e.g. 16% tricine (tricine running buffer) and 10% BisTris gels (MOPS running buffer), NuPage, Thermo Fisher Scientific, Waltham, MA, USA), but the overall procedure fits the method described by Laemmli (1970). The main principle of SDS-PAGE is separation of a protein mixture according to protein size (molecular weight) based on mobility in a polyacrylamide gel when applying an electrical field. Separation solely based on molecular weight is achieved by boiling the protein with SDS causing denaturation and masking of charges so that neither will have any influence on the migration in the gel. Information about protein molecular weights can then be obtained by comparing the generated band pattern with the pattern from a known standard.

3.3.3 Biotin switch

Two different procedures were tested for the detection of S-nitrosated peptides.

Initially detection of S-nitrosated peptides in the NCCPP aqueous ≤ 10 kDa extracts was conducted using a commercial S-Nitrosylated Protein Detection Kit from Cayman Chemical (Ann Arbor, MI, USA) based on the principles of the biotin switch (see Chapter 4.4.1). The experiment was performed in indirect light and according to manufacturer's instructions with some modifications. Briefly, a fixed volume of the samples were mixed 1:6 with Buffer A containing blocking reagent (thiol-blocking reagent) and incubated in the

dark, shaking at 300rpm for 30min at 7°C. Afterwards, total protein was precipitated in 4 volumes of pre-cooled acetone for 1 hour at -20°C. Following centrifugation at 3000g for 10min at 4 °C the pellet was re-suspended in 500µL Buffer B containing reducing and labelling reagent (biotin constituted the label) and further incubated in the dark for 1 hour at room temperature. Acetone precipitation was repeated as described above and the resulting pellet was re-suspended in 100µL of MilliQ water. In addition to aqueous ≤ 10 kDa extracts from the samples SH₀, DH₀ and SPS₀, h0/0 was also incorporated. MilliQ was included as negative control while cysteine and S-nitrosated cysteine was included as positive control. The S-nitrosocysteine was synthesised just prior to initiation of the assay by mixing equimolar concentration of cysteine and sodium nitrite dissolved in dH₂O and 0.1M HCl, respectively and incubating in the dark for 20-30min with gentle agitation.

Three µL of the treated/labelled aqueous ≤ 10 kDa extract samples and controls were dotted onto a polyvinylidene difluoride (PVDF) membrane which was dried under a blow-dryer, changing the air above the membrane from appropriate distance to limit heating. Free binding sites on the membrane were blocked overnight at 5°C in 2%(W/V) bovine serum albumin (BSA) in Tris-buffered saline (TBS; 20mM Tris-HCl, 0.8% NaCl, pH 8, diluted from 10x concentrated stock). The membrane was washed three times in TBS before incubating for 1 hour in S-Nitrosylation Detection Reagent I (horseradish peroxidase (HRP)) from the S-Nitrosylated Protein Detection kit diluted 1:75 in 2% BSA (W/V) in TBS. Again the membrane was washed three times in TBS and reactive signals were visualized using an Enhanced chemiluminescence (ECL) kit according to manufacturer's instructions (Amersham ECL Prime Western Blotting Detection Reagent, GE Healthcare Life Sciences, Chicago, IL, USA). Detection by substituting S-Nitrosylation Detection Reagent I (HRP) with S-Nitrosylation Detection Reagent II (Fluorescein) was also tested.

Alternatively the PierceTM S-Nitrosylation Western Blot kit from Thermo Fisher Scientific was applied. The experiment was conducted according to manufacturer's instructions with some modifications. In short, 100µL aqueous ≤ 10 kDa extract sample or control were mixed with 100µL HENS buffer (buffer composed of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), EDTA, neocuproine and SDS) and added 4µL of 1M methyl methanethiosulfonate (MMTS), vortexed for 1min and incubated for 30min at room temperature protected from light, in order to block free thiols. To remove excess MMTS proteins were precipitated with 6 volumes of cold acetone for minimum 1 hour at -20°C. The samples were centrifuged at 10,000g for 10min at 4°C, acetone decanted and the pellet was left to dry for 10min before resuspension in 100µL HENS buffer. The resuspended samples were split in two and both added 1µL 20mM Iodoacetyl Tandem Mass TagTM (iodoTMT) labelling reagent and vortexed to mix. Two µL 1M sodium ascorbate were added to all samples and positive controls but substituted for MilliQ in the negative control. The reaction was allowed to proceed for 1-2 hours. S-nitrosoglutathione and reduced glutathione were used as positive and negative controls, respectively.

The labelled samples were loaded onto a PVDF membrane as described above and free binding sites were blocked by incubating in 5%(W/V) skimmed milk powder dissolved in TBS with Tween-20 (TBST-20) for 1 hour at room temperature. The membrane was then incubated for 1 hour with the primary antibody (anti-TMT antibody diluted 1:1.000 in 5%(W/V) skimmed milk powder dissolved in TBST-20) and washed 5 x 5min in TBST-20. The membrane was then incubated with the secondary antibody (anti-mouse immunoglobulin-G HRP-conjugated antibody (IgG-HRP) diluted 1:40,000 in 5%(W/V) skimmed milk powder dissolved in TBST-20) at room temperature for 1 hour and subsequently washed 5 x 5min in TBST-20. The reactive signals were visualised using an ECL kit according to manufacturer's instructions (Amersham ECL Prime Western Blotting Detection Reagent, GE Healthcare Life Sciences).

3.3.4 Liquid chromatography-mass spectrometry (LC-MS)

The following method(s) were developed in close collaboration with experienced colleagues from the Research Group for Analytical Food Chemistry, at the National Food Institute.

Prior to LC-MS analysis the crude aqueous ≤ 10 kDa extracts of SH₀, DH₀, SPS₀ and h0/0 were subjected to further fractionation by ultrafiltration (UF). Vivaspin6 3000Da molecular weight cut off (MWCO) centrifugal concentrators (Sartorius, Göttingen, Germany) were rinsed with dH₂O according to manufactures description. Five mL of each aqueous ≤ 10 kDa extract were transferred to the rinsed centrifugal concentrators and centrifuged at 4000g (swing bucket) in short time intervals until approximately half the volume had passed the polyethersulfone (PES) UF membrane. The fractions were collected and returned to -80°C. The following LC-MS analysis of the ham extracts was performed on Dionex UltiMate 3000 LC system (Thermo Fisher Scientific) connected to a maXis electrospray ionisation-quadrupole time of flight (ESI-QTOF) mass spectrometer from Bruker (Bruker, Billerica, MA, USA).

Initially, 5 μ L of the ≤ 3 kDa extract fraction diluted 100x in dH₂O were injected into the system and analysed by flow injection with 50% B isocratic elution (A: 0.02% formic acid and 2.5mM NH₄OH B: methanol). Profiles were recorded using the mass range were 300-2900 m/z.

One μ L of the aqueous ≤ 10 kDa extracts, ≤ 3 kDa extract fractions, and concentrate from the UF were separated on a C8 column (Poroshell 120, 2.1x100mm, SB-C8 2.7 μ m, Agilent, Santa Clara, CA, USA) at 40°C in gradient set-up. Eluents A: 0.02% formic acid and 2.5mM NH₄OH and B: methanol running 0-1min 0% B, 1-3min 5% B, 3-10min 100% B, 10-12min 100% B, 12.1min 0% B and 12.1-14min 0% B at a constant flow rate 0.3mL/min. The following MS analysis was conducted in the mass range 300-2900 m/z with the following settings: end plate offset: 500V, capillary voltage: 4500V, nebulizer pressure: 2bar, drying

gas flow: 10L/min, dry gas temperature: 200°C and scan frequency: 9Hz. Nitrogen was used for all gas purposes.

To further investigate the low m/z ions, 1 μ L of the aqueous ≤ 10 kDa ham extracts from h0/0 and SPS₀ were analysed in a different chromatographic setup: eluent A: 0.1% formic acid in water and eluent B: 0.1% formic acid in acetonitrile, separation on C18 column (Poroshell 120, 2.1x100mm, SB-C18 2.7 μ m, Agilent) and a gradient (0-1min 1% B, 1-3min 15%B, 3-6min 50%B, 6-9min 95% B, 9-10min 95% B, 10.1min 1% B and 10.1-13min 1%B) at a constant flowrate of 0.4mL/min. The samples were detected in m/z range 50-1000. Mass spectrometer setting were kept the same except from the scan frequency which was changed to 2Hz. Broadband fragmentation were then conducted at energies 20-40eV.

Chapter 4

Results and Discussion

This Chapter is divided into five parts. Part A concerns antioxidative properties in commercial NCCPPs and is a summary of the main results, discussion and conclusions presented in Paper I and II. Meanwhile Part B concerns antioxidative properties in NCCPP model systems of varying nitrite and ascorbate addition and among others include a summary of the main results, discussion and conclusions from Paper III. Part C discusses the results from the investigations of antimicrobial activities (Paper I and III) as well as strategies for potential further investigations.

A series of experimental strategies were tested for further characterisation of the aqueous ≤ 10 kDa NCCPP extracts, and the results and challenges from this are presented in Part D. Lastly, Part E addresses some additional and supportive findings done during the PhD study.

4.1 Part A – Commercial NCCPPs

4.1.1 Characterisation of antioxidative properties in commercial NCCPPs (Paper I and II)

The characterisation of antioxidative properties in commercial NCCPPs were initiated by investigating whether antioxidant activity could be identified in aqueous ≤ 10 kDa extracts of different commercial NCCPPs.

Using *in vitro* assays for iron chelating activity, reducing power and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical scavenging activity, antioxidant activity was demonstrated for all three aqueous ≤ 10 kDa commercial NCCPP extracts in all assays with a single exception (Figure 4.1). The boiled dinner ham, DH, was generally the NCCPP demonstrating the poorest antioxidative properties and was also the one not demonstrating any iron chelating activity (Figure 4.1A).

With the exception of iron chelating, both reducing power and ABTS radical scavenging properties seemed to be connected with curing by way of all the aqueous ≤ 10 kDa nitrite-cured ham extracts displaying higher antioxidant properties than the reference, h0/0 (Figure 4.1). However, the reducing power and ABTS radical scavenging properties did not correlate with added nitrite/ascorbic acid but rather with residual nitrite/ascorbic acid (Table 3.2 in Chapter 3.1.1).

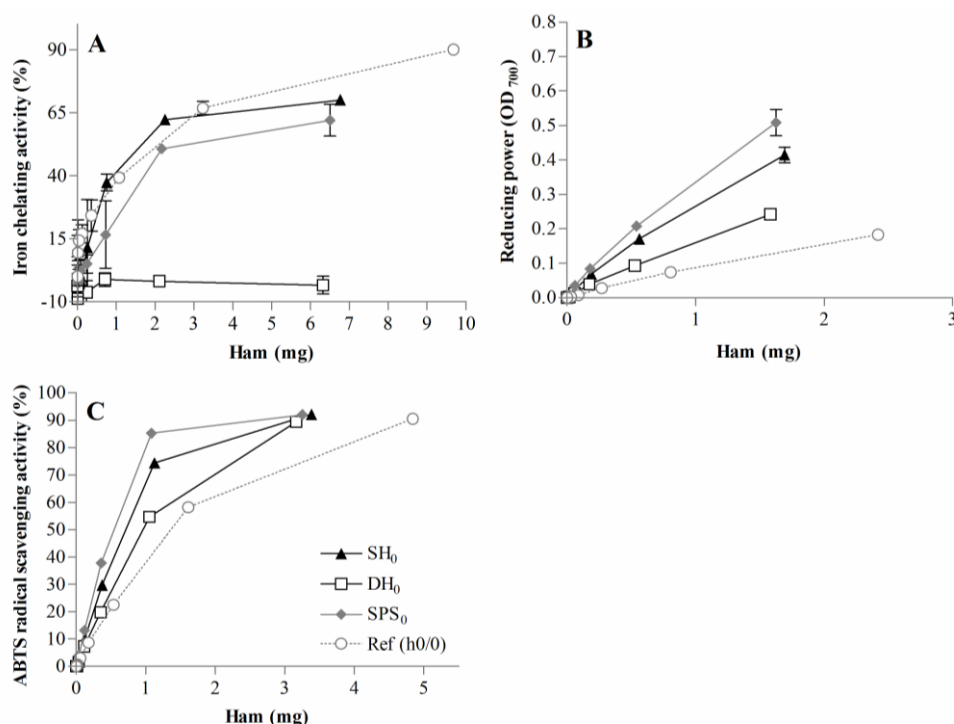


Figure 4.1 Antioxidant activities in the aqueous $\leq 10\text{kDa}$ extracts from the three commercial NCCPPs and the uncured reference ham (h0/0). A: Iron chelating activity (%) B: reducing power activity (OD_{700}) and C: ABTS radical scavenging activity (%). The activity of the extracts is plotted as function of mg ham used for making the analysed volume of aqueous $\leq 10\text{kDa}$ extract.

It is very reasonable to assume that any residual ascorbic acid may have been a great contributor to the detected reducing power and ABTS radical scavenging activity, as it is well known to be a strong reducing agent as well as a radical scavenger (Chapter 2.3.3). Yet, residual nitrite in itself was unlikely to have contributed greatly as nitrite mainly exerts its antioxidative activities through nitric oxide (NO) (Skibsted, 2011). Especially in the presence of a reducing agent such as ascorbic acid, it cannot be ruled out that the residual nitrite may function as a minute reservoir for NO or other nitrosating or antioxidant agents. In this way residual nitrite could have affected at least the detected ABTS radical scavenging activity.

The results furthermore indicated that the three types of detected antioxidant properties could not solely be ascribed to ascorbate and nitrite. Antioxidant peptides have previously been identified and purified from different dry-cured hams (Escudero, Mora, Fraser, Aristoy, & Toldrá, 2013; Mora *et al.*, 2014; Xing *et al.*, 2016). Thus, it was speculated that small peptides present in the aqueous $\leq 10\text{kDa}$ NCCPP extracts may have contributed to the detected antioxidant properties. Analysis of the amino acid composition of three commercial NCCPPs (and the uncured reference, h0/0) indicated a relatively high content of the known antioxidative proline and histidine, which could have resulted in the commercial NCCPPs exhibiting stronger antioxidative activity compared to the uncured reference ham.

All in all it was concluded that the currently demonstrated antioxidant activity in the aqueous ≤ 10 kDa extracts must have been caused by strong synergetic effects of protein and non-protein, anti- and pro-oxidant compounds originating from processing and raw materials.

In order to further investigate the potential origin of antioxidant activity from other compounds than residual ascorbate and nitrite in the aqueous ≤ 10 kDa NCCPP extracts, the crude extracts were separated by size exclusion chromatography (SEC) and the collected fractions were analysed using the same *in vitro* antioxidant assays. In the attempt to confine potential interferences, nitrite was measured at several time points during storage of the commercial NCCPPs to identify the time point at which residual nitrite reached a low level, e.g. ensuring that formation of nitrite related reaction products would have reached its maximum/steady level. This occurred after 37 days of storage.

The three aqueous ≤ 10 kDa commercial NCCPP extracts all had very similar SEC profiles (Figure 4.2) which were characterized by four main SEC peaks (I: 18.5-19.9mL, II: 19.9-21.4mL, III: 21.4-22.9mL and IV: 25.3-27.2mL). Despite all the sample material being nitrite-cured cooked hams the SEC profile demonstrated slight inter and intra peak differences regarding absorbance intensity (Figure 2 in Paper II). This could be a result of the hams being different in respect to meat cut, whole muscle vs. smaller pieces, type and amount of additives and manufacturing procedure, e.g. smoking (SPS). A selection of peptides and amino acids were also subjected to SEC separation and their point of elution is marked in Figure 4.2. The elution of cytochrome C (CytC) and aprotonin long before the sample SEC peaks indicated the aqueous ≤ 10 kDa extract constituents to be smaller than 6.5kDa (size of aprotonin).

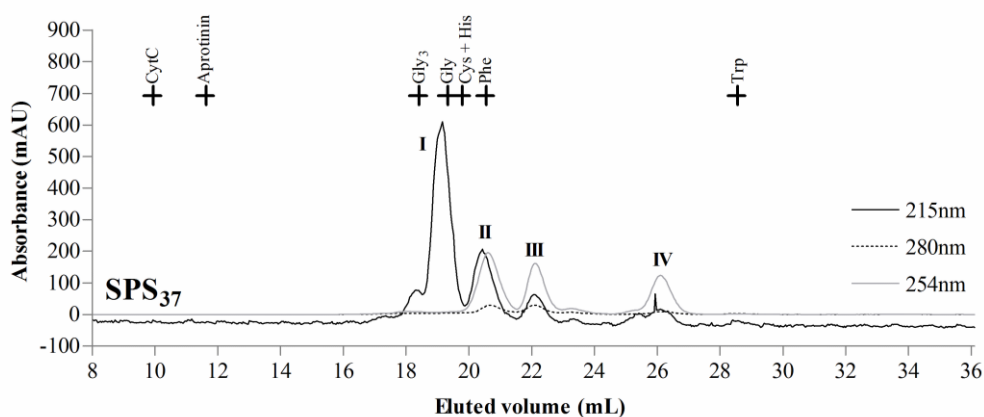


Figure 4.2 Representative SEC chromatogram of the aqueous ≤ 10 kDa extracts of the smoked pork saddle after 37 days of storage (SPS₃₇). The elution of the injected external standards (peptides and amino acids) is marked at the top.

After several attempts of optimization it was clear that the iron chelating assay was not compatible with the low concentrations of sample constituent occurring after the chromatographic separation and nor with the

0.1M ammonium acetate used as chromatographic eluent. Thus, the characterisation was continued with only two antioxidant assays.

The *in vitro* antioxidant activity was concentrated to only a few fractions from the SEC separation (Figure 4.3). Reducing power activity (Figure 4.3A) was concentrated around to fraction-peaks, P1 and P2, which coincided with the elution of SEC peak I and II, respectively. ABTS radical scavenging activity was measured after 1min incubation (Figure 4.3B) and 31min incubation (Figure 4.3C). After 1min incubation activity was detected in fraction-peak, P3 (21.1-23.1mL), and after the additional 30min incubation ABTS radical scavenging activity also appeared in the fraction-peak, P4, (18-20mL). The arising of this fraction-peak after the additional incubation clearly showed different reaction kinetics of the mixture of active compounds enclosed in P3 and P4. P4 aligned with P1 and thus, also aligned with the SEC peak I (Figure 4.2). Yet, it was shifted slightly to the right while P3 aligned nicely with peak III.

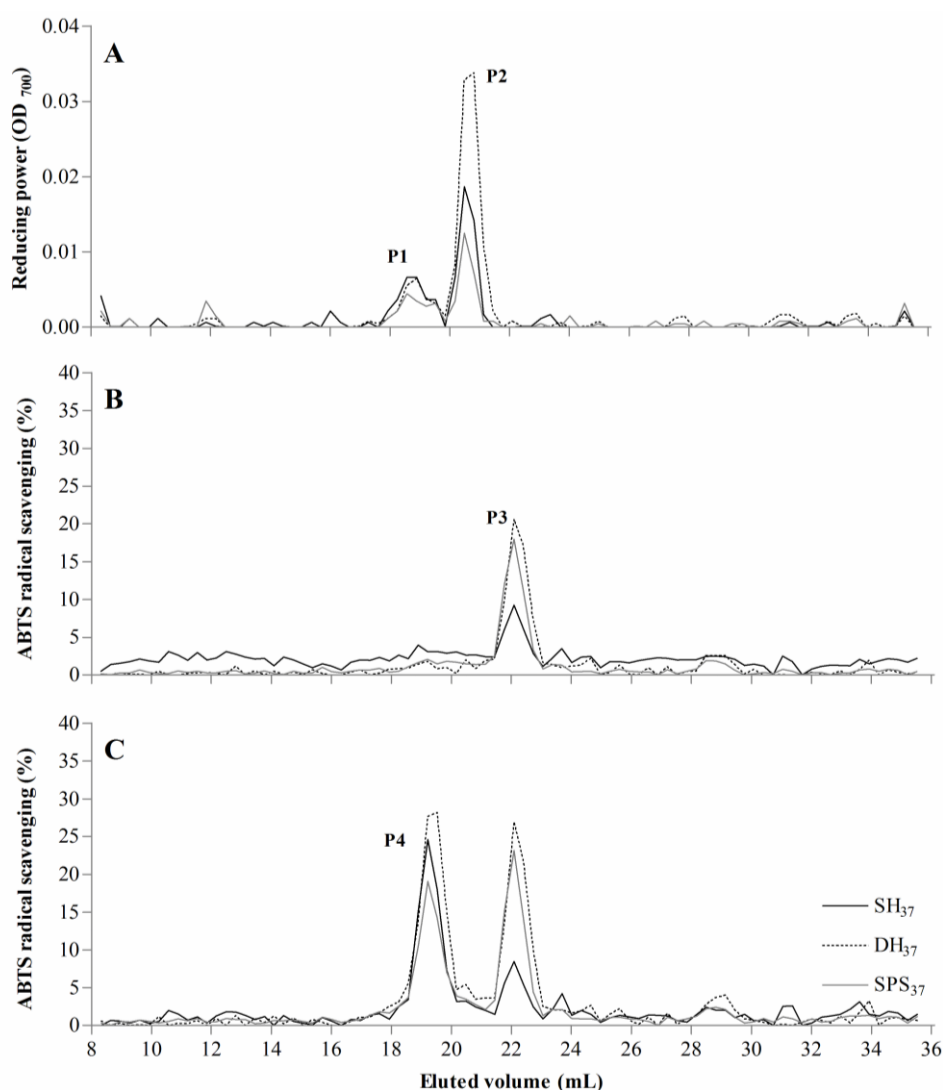


Figure 4.3 Antioxidant behavior of SEC fractions measured using *in vitro* assays for A: reducing power, and ABTS radical scavenging after B: 1min and C: 31min of incubation.

A comparative analysis of the SEC profiles, elution of the external markers (peptide and amino acid), active antioxidative SEC fractions, protein content as well as total and free amino acid profiles of the extracts were conducted in the attempt to explain the origin of the observed antioxidative activities (Paper II). The absorbance patterns in the SEC profiles indicated peak I to contain small peptides but potentially also free amino acids other than the aromatic: phenylalanine, tyrosine and tryptophan. Normalisation of antioxidant activity to proteins content in this fraction left an impression that the reducing power in P1 might be connected the small peptides. This was, however, not obvious for the ABTS radical scavenging activity in P4. The free amino acid profile did not offer a clear explanation either. However, the slower reaction kinetics of P4 compared to P3 was also observed for solutions of histidine, proline and glycine when analysed in concentration $>0.02\text{mg/mL}$, but not at concentration equivalent to the content of the individual free amino acids in the extracts (see Chapter 4.5.2). The analysis pointed towards SEC peak II containing small peptides as well as phenylalanine either free or embedded in peptides. Both may have contributed to the detected reducing power activity in fraction-peak P2, although the literature primarily reports phenylalanine to be of importance in radical scavenging (Duan *et al.*, 2014; Sarmadi & Ismail, 2010). The activity in P3 was also speculated to be the action of peptides or a compound containing some sort of aromatic ring. This could for instance have been tyrosine as the elution of the other aromatic amino acids, phenylalanine and tryptophan, was already established (Figure 4.2). Yet nothing conclusive could be said on this matter as solubilisation and therefore injection of free tyrosine was unsuccessful (Chapter 4.5.2). Solutions of tyrosine (although turbid) have been found to be highly potent at colour reduction even at low concentrations (15-22% ABTS radical scavenging activity at 0.002mg/mL , see Chapter 4.5.2). Though it appeared that a peptide containing tyrosine could contribute to the *in vitro* ABTS radical scavenging activity, the degree of importance in a NCCPP matrix may be slightly reduced, as studies have shown that tripeptides with C-terminal tyrosine or tryptophan are weak scavengers of peroxynitrite (ONOO^-) (Eq. 13 in Chapter 2.3.3)(Saito *et al.*, 2003).

No clear correlation could be extrapolated from the distribution of total and free amino acids in the extracts. However, the extracts were dominated by histidine, alanine, methionine, glutamic acid and glycine in decreasing order. Both histidine and methionine are among the amino acids frequently mentioned in regard to peptide related antioxidant activity and thereby supported the expectation of finding higher antioxidant activity in the aqueous $\leq 10\text{kDa}$ fraction compared to the entire ham. It is also noteworthy that carnosine (226.24Da), the dipeptide found in meat possessing antioxidant activity (Chapter 2.6), is made up of exactly alanine and histidine.

Although, the extracts are expected to contain a vast variety of compounds that could contribute to the detected antioxidant properties, these results emphasize the importance of peptides for antioxidative properties in NCCPPs.

4.2 Part B – Model systems

4.2.1 Antioxidant activity in a ham model system (Paper III)

The following paragraph summarises the main findings on antioxidant activity from the third manuscript (Paper III).

As previously described it is common practice to add a combination of nitrite and ascorbate when curing meat. Ascorbate as an electron donor, is an effective antioxidant (Bendich, Machlin, Scandurra, Burton, & Wayner, 1986) and although nitrite, as a natural electron acceptor, can be considered a strong prooxidant, it is well established that their combined addition inhibits lipid oxidation (Skibsted, 2011). Although, the redox chemistry of nitrite and ascorbate is essential in nitrite-curing and extension of shelf-life it is highly complex and at times not fully understood. Thus, in order to investigate the impact of the curing agents in question on *in vitro* antioxidant activity a set of model hams with addition of parallel increasing levels of nitrite and ascorbate were produced (see Chapter 3.1.2). The effect of cold-storage of the hams was included in the investigations in order to put the findings in a shelf-life extending perspective.

Both ascorbate and nitrite are highly reactive compounds, and their reaction products may have altered properties in regard to antioxidant activity. Thus, it is of the essence to track changes in residual levels of the two compounds during cold storage (Figure 4.4).

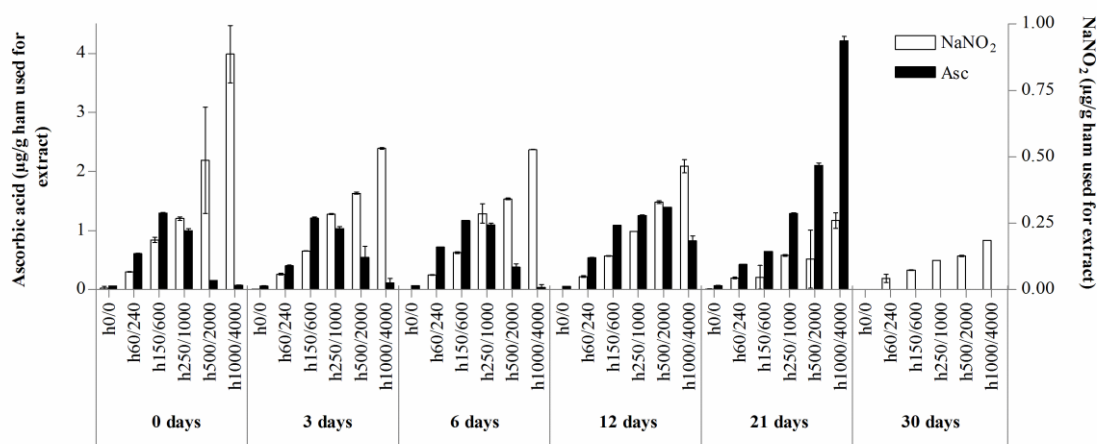


Figure 4.4 Average sodium nitrite and ascorbic acid content in the crude aqueous ≤ 10 kDa extracts made from the model hams at all concentration levels stored for 0, 3, 6, 12, 21 and 30 days at 5°C. The content has been normalised to the amount of ham utilised for the meat/water homogenate for producing the extracts. (n=2). Residual ascorbic acid was not measured at day 30 due to a technical error.

The observed decrease in residual nitrite during cold storage has often been reported (Pérez-Rodríguez, Bosch-Bosch, & García-Mata, 1996). However, the detection of lower levels of ascorbic acid in samples of high addition early in storage followed by residual ascorbic acid increasing with ascorbate addition late in storage was highly unexpected and no clear explanation was available.

All aqueous ≤ 10 kDa model ham extracts, including the reference (h0/0), demonstrated all three tested forms of antioxidant activity; ABTS radical scavenging activity, reducing power and iron chelating activity, at some point in storage and dilution.

The ABTS radical scavenging activity increased with extract concentration and eventually appeared to approach the assay maximum of approximately 90% (as was observed for the commercial NCCPPs). This occurred for all extracts throughout storage (data not shown). Thus, in order to see a potential effect of curing agents and storage it was decided to focus on the linear activity increase in the initial/low extract concentration part of the dilution curves – the normalised ABTS radical scavenging activity (Figure 4.5)

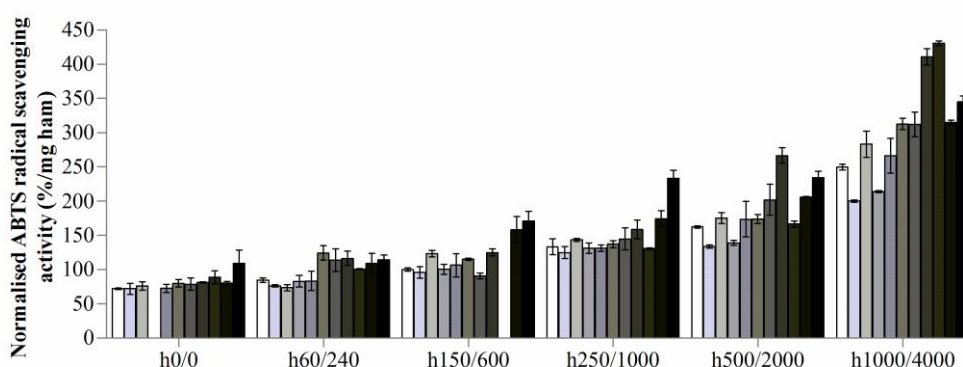


Figure 4.5 Normalised ABTS radical scavenging activity (%/mg ham) in the crude ≤ 10 kDa model ham extracts during storage distributed on nitrite/ascorbate addition. The white bar indicates 0 days of cold storage and for each shade darker +3 days of storage until the black bar indicating 30 days of cold storage. Sample h0/0 day 9 and h150/600 day 24 were lost.

From Figure 4.5 it was evident that a positive correlation between normalised ABTS radical scavenging activity and storage time as well as added nitrite/ascorbate existed. At day 0 (white bars in Figure 4.5) normalised ABTS radical scavenging activity also correlated with residual nitrite, as residual and added nitrite converged. However, the increase in normalised ABTS radical scavenging activity occurring over time (Figure 4.5) was contrary to the decrease in residual sodium nitrite happening over time (Figure 4.4 - higher added sodium nitrite still resulted in higher residual sodium nitrite at any time point during storage). As nitrite do not primarily exert its versatile antioxidant properties directly as nitrite but through different reaction products (Freyblyer *et al.*, 1993; Honikel, 2008; Skibsted, 2011), it can be assume that the decrease in residual nitrite over time is resulting in formation of more antioxidant species, and that a higher addition of nitrite provides more starting material for such a conversion. On contrary, normalised ABTS radical scavenging activity did not correlate with residual ascorbic acid until the end of the storage period.

The aqueous ≤ 10 kDa extracts of the model hams stored at -80°C directly from production (day 0) demonstrated some unexpected reducing power activity results (Figure 4.6). Reducing power activity in the three extracts of the model hams with the lowest nitrite/ascorbate addition increased with extract

concentration. Meanwhile the extracts of model hams with the three highest levels of nitrite/ascorbate only increased with concentration at low extract concentrations however decreased at higher extract concentrations to an extent, where no reducing power were demonstrated in the crude aqueous $\leq 10\text{kDa}$ extract of the sample added the most nitrite and ascorbate (h1000/4000)(see Figure 4.6). However, after 3 days of storage the reducing power activity of the h1000/4000, h500/2000 and h250/1000 crude aqueous $\leq 10\text{kDa}$ extracts started to surpass the reducing power activity of the three low nitrite/ascorbate samples. After 12 days of cold storage the measured crude aqueous $\leq 10\text{kDa}$ extract reducing power activities directly correlated with added nitrite/ascorbate (see Figure 2 in Paper III). The reducing power activity tended to correlate with residual ascorbic acid.

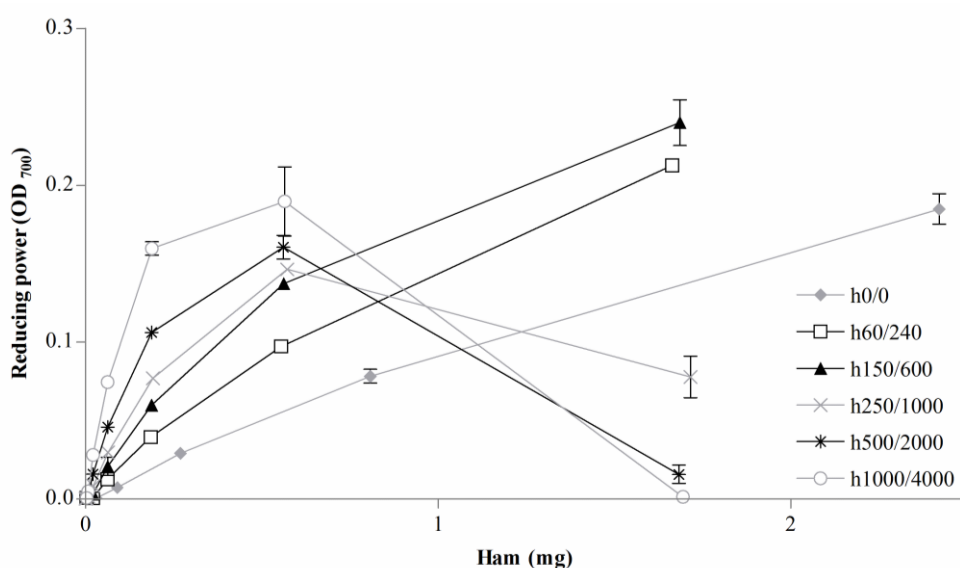


Figure 4.6 Reducing power (OD_{700}) dilution curve for the aqueous $\leq 10\text{kDa}$ model ham extract measure after 0 days of storage ($n=3$ but $n=6$ for h250/1000, h500/2000, h1000/4000). “Ham (mg)” on the x-axis signifies mg ham used for the meat/water homogenate used for producing the extract.

Regardless of this deflection of the dilution curves detected early in storage for samples of high nitrite/ascorbate addition, the initial linear reducing power activity as function of mg ham increased with the added ascorbate/nitrite for all samples. However, there were no significant correlation between the initial linear reducing power activity and time of storage – only a tendency.

It was speculated that the large amounts of ascorbate added to the three samples demonstrating the deflection caused an overload of the reducing power assay. The iron rich detection compound, Prussian blue, is formed upon reduction of iron (Fe^{3+}) but additional reduction potentially caused by the strong reducing agent (e.g. ascorbate) would cause it to return to colourless. However, this explanation is contradictive to the amount of residual ascorbic acid in the samples (Figure 4.4). On the other hand, the reducing power activity of the crude aqueous $\leq 10\text{kDa}$ model ham extracts at day 0 showed some resemblance with the residual ascorbic

acid in regard to the drop occurring at additive concentration above 150 ppm sodium nitrite and 600ppm sodium ascorbate. The relationship between residual ascorbic acid and reducing power of the crude aqueous $\leq 10\text{kDa}$ model ham extracts continued during storage with a slight time discrepancy.

In spite of ascorbic acid being capable of chelating metals (Brady, 2013) and some of the proposed antioxidant activities of nitrite involve chelating of metals (Igene *et al.*, 1985) no unambiguous correlation between added or residual ascorbate/nitrite and iron chelating activity was found at any point during storage. Thus, it must be assumed that the detected iron chelating activity originated from other ham components in the aqueous $\leq 10\text{kDa}$ extracts and the slight differences observed between the samples could stem from differences in the utilised pork meat e.g. proteins or amino acids rather than additives. A weak forming tendency to increasing iron chelating activity with storage time in samples of higher nitrite/ascorbate addition inspired the hypothesis that nitrite/ascorbate addition, beyond a certain threshold concentration, (indirectly) affect a time depended formation of an active component(s).

4.2.2 Antioxidant activity in a sausage model system

A small study was set up to evaluate the impact of shifted nitrite and ascorbate addition to comminute wiener sausages on *in vitro* antioxidant activity. A full matrix experiment was drafted (see Chapter 3.1.3) but in order to limit the extent of laboratory work this initial investigation was restricted to include the sample extremes and the matrix centre: s0/0, s0/2000, s105/240, s500/0 and s500/2000. The aqueous $\leq 10\text{kDa}$ fraction was extracted and diluted threefold as described in Paper I & III and tested for iron chelating, reducing power and ABTS radical scavenging activity. This was only meant as a preliminary study yet some noteworthy tendencies were evident which are worth commenting on.

The ABTS radical scavenging activity was clearly affected by the amount of ascorbic acid added to the model sausage (Figure 4.7). The same correlation as observed in model hams (Paper III). However, looking at s0/2000 and s500/2000 which were added the same amount of ascorbic acid the sample also added nitrite increased in ABTS radical scavenging activity with extract concentration at a slower rate. Thus, it may be speculated that the combined addition of the two curing agents had resulted in their mutual interactions, leading to a decrease in both compounds in the free form overall causing the detected *in vitro* ABTS radical scavenging activity to decrease. Residual ascorbic acid was determined for the sausages and aqueous $\leq 10\text{kDa}$ sausage extracts but the variation and uncertainty of the results were too grand to support any further speculation on the importance of interactions between the two curing agents. The great resemblance between s0/0 and s500/0 indicated limited effect of added nitrite alone on ABTS radical scavenging. For the aqueous $\leq 10\text{kDa}$ model ham extracts in Paper III both added and residual nitrite (as the converged) were found to correlate with the normalised ABTS radical scavenging activity. However, it must be kept in mind that all

the model hams added nitrite were also added ascorbate. Residual nitrite was not expected to have an impact as nitrite primarily exerts its antioxidant properties through reactions of NO (Freybler *et al.*, 1993; Honikel, 2008; Skibsted, 2011). This was furthermore confirmed in the context of the currently applied assay as solution of sodium nitrite did not result in any reduction in colour formation in the ABTS radical scavenging assay. In addition, these results further substantiated the presence of unidentified compounds resulting in a basal level of radical scavenging properties in cooked pork products, as ABTS radical scavenging activity was now identified in both comminute sausages and restructured hams not added curing agents (Paper III).

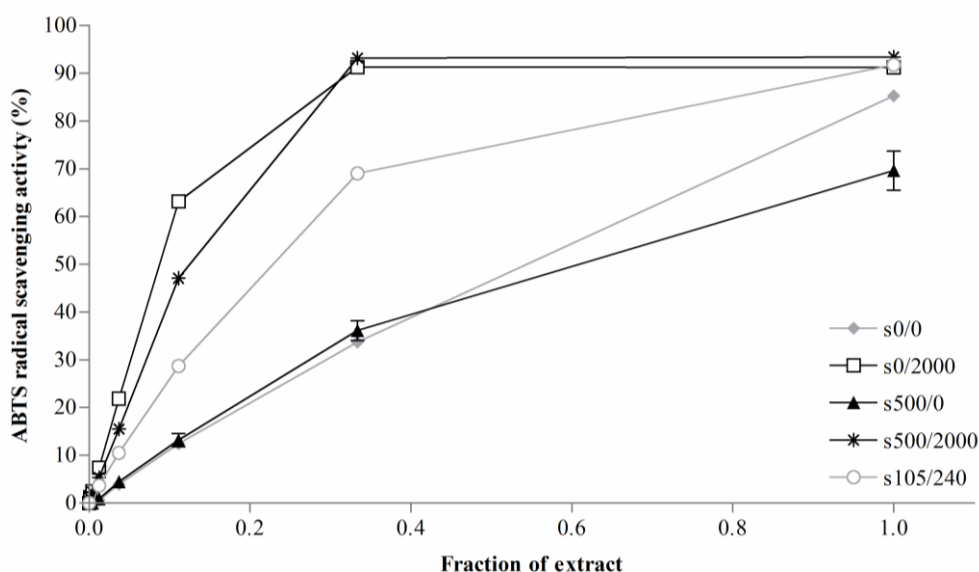


Figure 4.7 ABTS radical scavenging activity (%) dilution curve for the tested aqueous $\leq 10\text{kDa}$ model sausage extracts expressed as function of the fraction of the crude aqueous $\leq 10\text{kDa}$ extract.

The dilution curves for the reducing power activity of the aqueous $\leq 10\text{kDa}$ extracts of the five tested model sausages are presented in Figure 4.8. The dilution curve of s500/2000 showed the same defective behaviour beyond a certain extract concentration as was also observed for h500/2000 in Figure 4.6 and Figure 3 in Paper III. In the aqueous $\leq 10\text{kDa}$ extracts of the model hams a connection with added ascorbate beyond a certain threshold was pointed out though in the current experiment the sample with 2000ppm added ascorbic acid but no added nitrite (s0/2000) showed an almost linear increase with extract concentrations ($R^2=0.998$). Thus, it would appear that the deflection in the reducing power dilution curve might be related to an interaction between ascorbic acid and nitrite rather than a double reduction of iron in connection with Prussian blue due to high addition of the strong reducing agent (ascorbate) as mentioned in Chapter 4.2.1 and Paper III. This interaction between nitrite and ascorbate could likely be affecting the residual ascorbic acid content which in Paper III was discovered to correlate with the reducing power activity of the crude aqueous

≤ 10 kDa extract. As mentioned above the measurement of residual ascorbic acid in the aqueous ≤ 10 kDa sausage extracts was erroneous and thus cannot support or reject the proposed explanation.

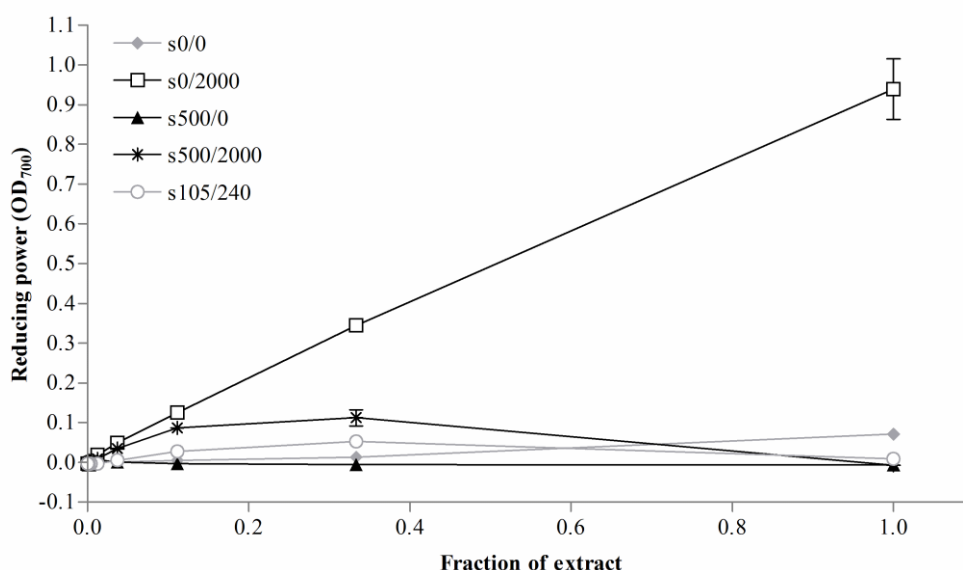


Figure 4.8 Reducing power (OD₇₀₀) dilution curve for the tested aqueous ≤ 10 kDa model sausage extracts expressed as function of the fraction of the crude aqueous ≤ 10 kDa extract.

Surprisingly s105/240 also deflected at high extract concentrations. This result is curious if assuming that added ascorbic acid was the sole explanation as neither h60/240 nor h150/600 (with the same or more added ascorbate) behaved in this manner (Figure 4.6 and Figure 3 in Paper III). The main difference between the mentioned model hams and the model sausage is the added nitrite to added ascorbate/ascorbic acid ratios of 1:4 and 1:2.3 respectively. Thus, it would appear that along with some unknown threshold value the ratio is essential for this deflecting behaviour. Furthermore, it would seem that the amount of nitrite is defining as the deflection only occurs when nitrite is added but only when ascorbate is added too. This was emphasized by the complete lack of OD₇₀₀ signal in s500/0. This result shows that great attention should be paid to the formulation of NCCPPs as slight changes in the amount of added curing agents may change the preservative effect.

Like it was observed in the aqueous ≤ 10 kDa extracts of the model hams, s0/0 also displayed some reducing power activity (Figure 4.8). Thus, it would seem that cooked pork products in general may have a basic level of reducing power activity just as was seen for ATBS radical scavenging activity. This could potentially originate from the meat e.g. peptides and free amino acids. This is, however, largely overshadowed by the ascorbate/nitrite chemistry in the current experimental setup.

The analysis of *in vitro* iron chelating activity of the aqueous ≤ 10 kDa model sausage extracts revealed no obvious correlations.

Although, only preliminary these investigation clearly underlines the importance of the interplay and interactions of the two curing agents for *in vitro* reducing power and ABTS radical scavenging activity.

4.2.3 SEC on extracts from model hams and sausages and antioxidant capacity of the SEC fractions

As an extension of the investigation of the connection between nitrite/ascorbate and *in vitro* antioxidant activity in NCCPPs, the aqueous ≤ 10 kDa extracts of the model hams (parallel increasing nitrite and ascorbate addition) used in Paper III and the model sausages (shifted nitrite and ascorbate addition) described above were attempted further characterised by means of SEC and *in vitro* antioxidant activity assays on the collected SEC fractions. It was ascertained in a similar experimental set up in connection with Paper II that the iron chelating activity assay was not compatible with SEC fractionation. For this reason only reducing power and ABTS radical scavenging activity was measured and included in the present characterisation. In addition to enlightening the dependence of nitrite/ascorbate for *in vitro* antioxidant activity these result would also complement the findings, discussion and conclusion made in Paper II.

SEC

The SEC chromatograms of both model hams and sausages (Figure 4.9) contained 4 main peaks eluting at the same time points as the 4 main peaks (I, II, III and IV) presented in Paper II and Figure 4.2 in Chapter 4.1.1. Consequently, it can be assumed that the model ham and sausage aqueous ≤ 10 kDa extracts like the aqueous ≤ 10 kDa commercial NCCPP extracts only contained compounds ≤ 6.5 kDa. Furthermore, it can be postulated that any pork product (whole muscle, restructured or minced) subjected to nitrite-curing and more importantly heat-treatment only contain compounds ≤ 6.5 kDa in their aqueous low molecular weight fraction. Later analysis has indicated that the constituent water soluble compounds might in fact be even smaller (See Chapter 4.3.2 on LC-(ESI)-MS).

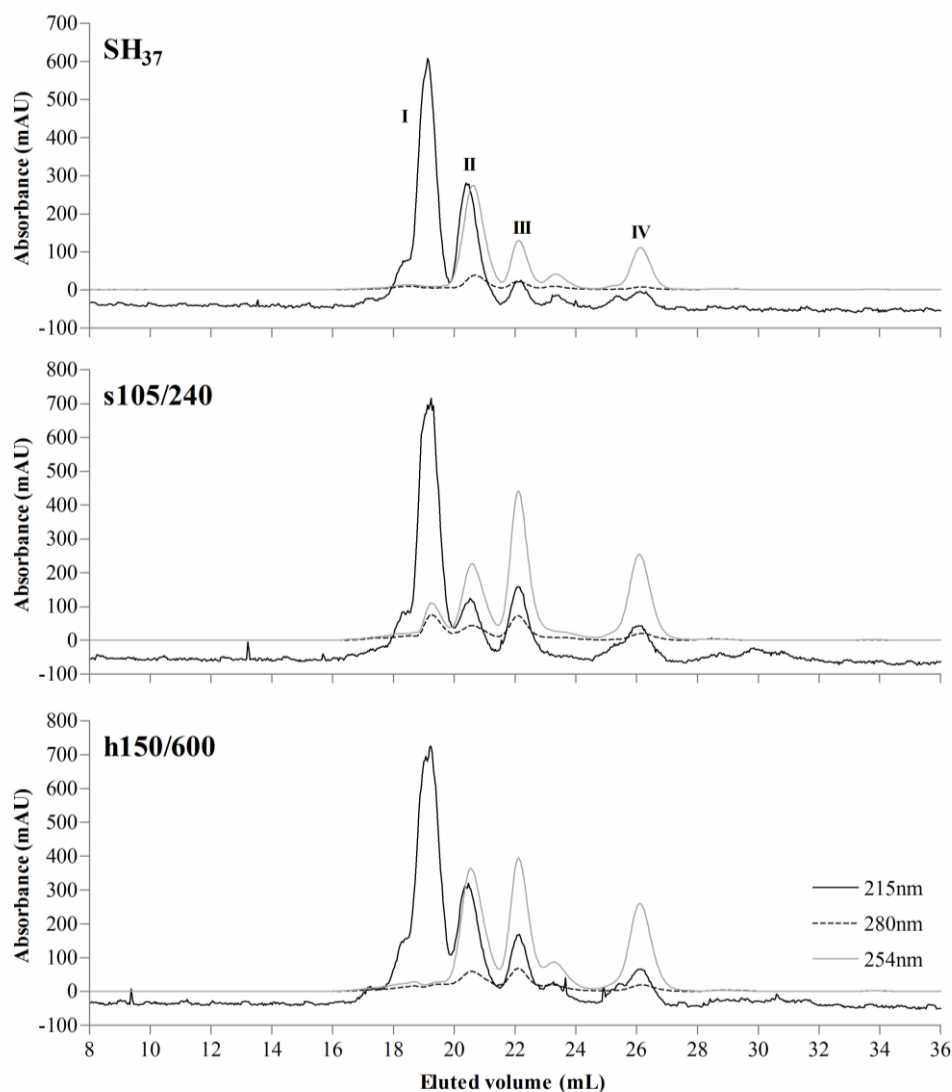


Figure 4.9 Comparison of representative SEC profiles of aqueous ≤ 10 kDa extracts from commercial NCCPPs and model hams and sausages. SH₃₇: the commercial restructured sandwich ham, s105/240: model sausage added 105 ppm sodium nitrite and 240 ppm ascorbic acid and h150/600: model ham added 150 ppm sodium nitrite and 600 ppm sodium ascorbate.

Slight differences were, however, present between the two sample categories. In Figure 4.9 a small peak, not fully baseline separated, eluted just after SEC peak III in the commercial NCCPPs and model hams but not in the sausages. The occurrence and intensity of this peak did not correlate with nitrite and ascorbate addition. In Paper II it was argued that any differences in inter-peak intra-chromatogram signal magnitude between the three tested aqueous ≤ 10 kDa commercial NCCPP extracts was likely due to product differences in regard to processing and starting material. This hypothesis was partly supported as the inter-peak relationship was the same for all model hams (215nm: I>II>III>IV) and all model sausages (215nm: I>III>II>IV) independent of nitrite/ascorbate addition. It would seem that processing might have a larger influence as the sausages were produced at the same time from the same batch of minced meat while the model hams were produced in two

rounds although according to the same procedure. The stable inter-peak relationship identified from the 215nm absorbance curve in the model products was not as clear from the 280nm and 254nm absorbance curves. The absorbance at these wavelengths increased in SEC peak I with increasing ascorbate addition (Figure 4.10). This was evident both in the hams with parallel increasing nitrite/ascorbate addition and sausages only added ascorbic acid or added both ascorbic acid and nitrite

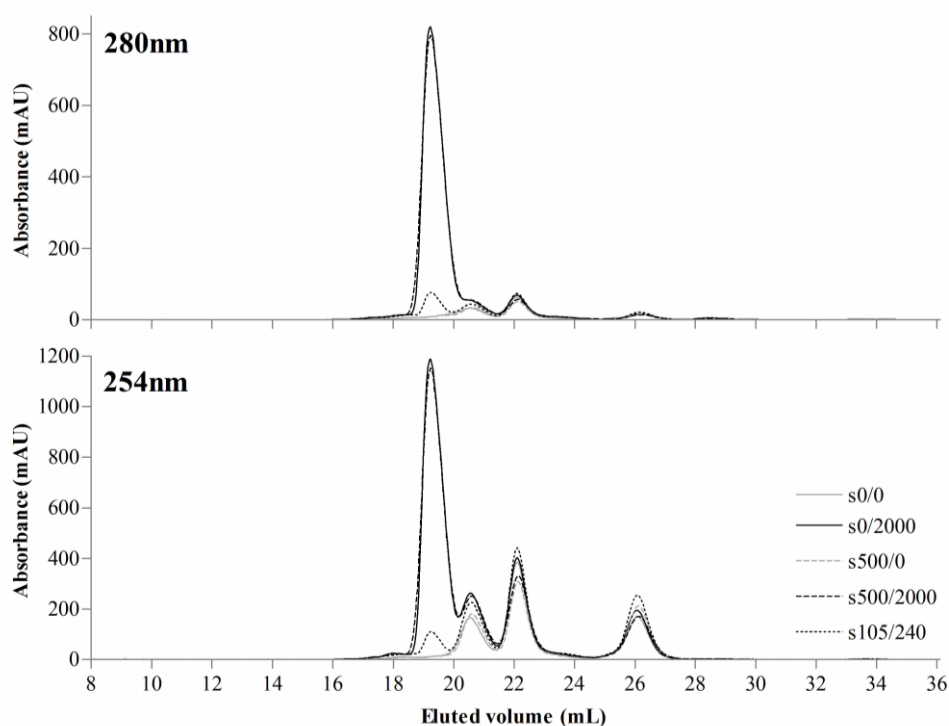


Figure 4.10 The effects of added ascorbate on peak I during SEC separation measured at 280nm and 254nm. The spectrums were recorded on aqueous $\leq 10\text{kDa}$ sausage extracts.

Below an unidentified threshold of nitrite/ascorbate addition the intensity of the signal (280nm and 254nm) in SEC peak I decreased relatively fast. The two hour time interval between injections onto the fast protein liquid chromatograph was long enough to have a clear impact on SEC peak I in sausage sample s105/240 (Figure 4.11). The same effect was seen in the h60/240 and h150/600 model ham samples. At higher concentration of nitrite/ascorbate addition the intensity of SEC peak I did not decrease within the analysis timeframe. However, a specimen of the h500/2000 model ham was by mistake left at 5°C for 276 days and subjected to extraction with dialysis (referred to as h500/2000₂₇₆). The much longer time of cold storage (although of the ham and not the aqueous $\leq 10\text{kDa}$ extract like for e.g. s105/240) resulted in a decrease in 280nm/254nm signal intensity compared to the h500/2000 ham extracted without preceding cold storage (data not shown).

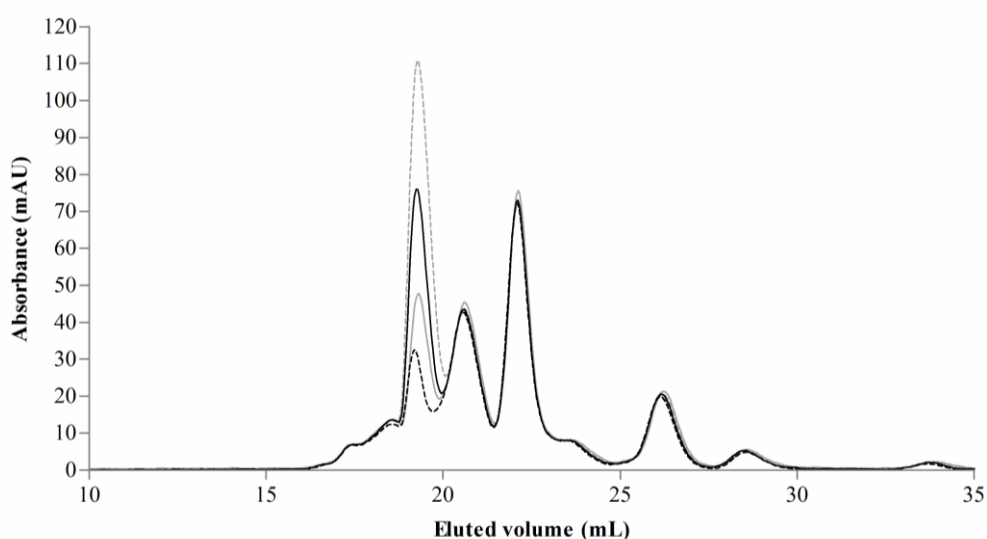


Figure 4.11 Changes in absorption at 280nm in peak I during SEC fractionation of the aqueous ≤ 10 kDa extract from model sausage s105/240. The four injections were approximately 2 hours apart and the samples were stored at 5°C in the meantime. The decrease was also visible at 254nm.

Ascorbic acid is a rather sensitive compound. Thus, the decrease in SEC peak I over time could be an argument for the 280/254nm signal being largely induced by the labile ascorbate. Despite high levels of uncertainties compromising the credibility of the results the aqueous ≤ 10 kDa extract of s0/2000 were found to contain more residual ascorbic acid than s500/2000 which also contained more residual ascorbic acid than s105/240, all of which correlated with the 280/254nm signal intensity. However, the correlation was not linear. On contrary the residual ascorbic acid content was in Chapter 4.2.1 Figure 4.4 found not to increase continuously with nitrite/ascorbate addition and furthermore, residual ascorbic acid increased with cold storage for extracts of h250/1000, h500/2000 and h1000/4000 (Paper III). Both arguments are opposing the hypothesis of SEC peak I originating mainly from ascorbic acid. As the results point in opposite directions a final conclusion on the importance of ascorbate for the 280/254nm signal cannot be drawn.

In the sausage sample with added nitrite but without added ascorbate (s500/0) a clear peak eluted after SEC peak IV only absorbing at 215nm (Figure 4.12). This 215nm peak was also discovered in the remaining sausage and ham samples added ≥ 500 ppm nitrite. However, the addition of ascorbate appeared to result in a lowering of the signal. Nitrite and nitrate has low-wavelength absorptivity and wavelengths between 205-215nm are frequently used for their detection (Zacharis & Tzanavaras, 2012). Thus, this peak could be associated with nitrite/nitrate. However, injections of nitrate solutions resulted in a signal responds at approximately 19mL (data not shown). Yet, the reduction of the peak with ascorbate addition fits the general notion that residual nitrite decreases faster in the presence of ascorbate (Izumi, 1992).

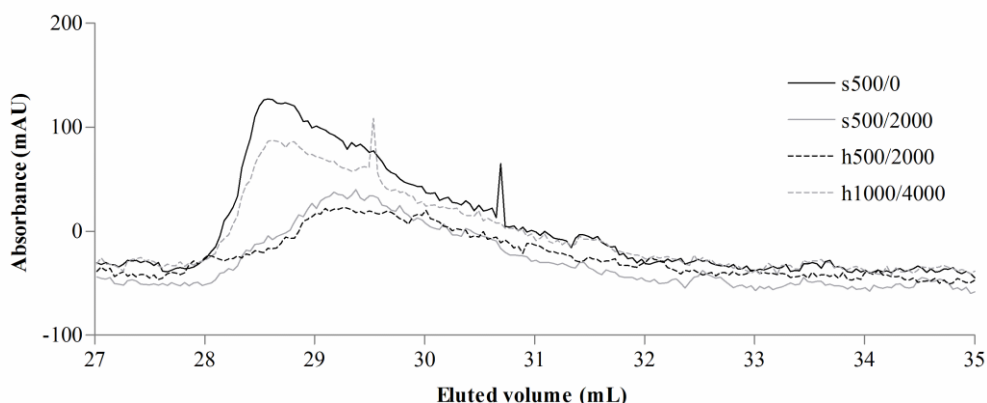


Figure 4.12 Absorption at 215nm during SEC fractionation of aqueous $\leq 10\text{kDa}$ extracts from model hams and sausages added $\geq 500\text{ppm}$ nitrite.

Antioxidant activity

The SEC fractions of the aqueous $\leq 10\text{kDa}$ sausage extracts only demonstrated reducing power activity in extracts of sausages added ascorbate which is a known strong reducing agent. The results from the sausages left the impression that reducing power activity in NCCPPs might solely be connected with ascorbate which was contradictory to the clear OD_{700} signal appearing in h0/0 with no added ascorbate (naturally occurring residual ascorbic acid was $1.1\mu\text{g/mL}$ extract). All SEC fractionated model ham aqueous $\leq 10\text{kDa}$ extracts exhibited *in vitro* reducing power activity. The course of the activity curves was divided in two fraction-peaks which was located as for the commercial NCCPP $\leq 10\text{kDa}$ extract samples in Paper II and Figure 4.3 in Chapter 4.1.1 (P1 and P2). Some correlation with ascorbate was also evident in the model hams. As was also the case for the aqueous $\leq 10\text{kDa}$ commercial NCCPP extracts, P1 was broad and wavy and could be perceived as a “double peak” (not baseline separated) peaking after 18.5mL (P1.1) and 19.5mL elution (P1.2). As ascorbate additions increased so did OD_{700} max for P1.2. The sausage P1 was not wavy as for the model hams but also peaked at 19.5mL and was also broad and occasionally overtook the very much weaker fraction-peak P2. The large P1 in the sausages tended to decrease with nitrite addition (Figure 4.13) as also demonstrated in the crude aqueous $\leq 10\text{kDa}$ sausage extracts. This tendency could, however, not be deduced from the model ham results as added nitrite/ascorbate as well as added and residual nitrite converged. This decrease with nitrite addition may be related to the increased metabolism of nitrite as a consequence of ascorbate addition, which will also lead to increased “consumption” of ascorbate. This means that the reducing power activity detected in the fractions of the aqueous $\leq 10\text{kDa}$ sausage extracts were connected with residual ascorbic acid rather than added ascorbate. The same relationship between residual ascorbic acid and reducing power was also demonstrated for the aqueous $\leq 10\text{kDa}$ model ham extracts (Paper III) – a relationship which, as stated above, changed after SEC fractionation from correlating with residual ascorbic

acid to correlating with added ascorbate. As was the case for the SEC fractionated aqueous $\leq 10\text{kDa}$ commercial NCCPP extracts in Paper II the SEC peak I and P1 also aligned for the extracts of model hams and sausages. The intensity of the 280/254nm signal in peak I correlated with the reducing power activity in P1.

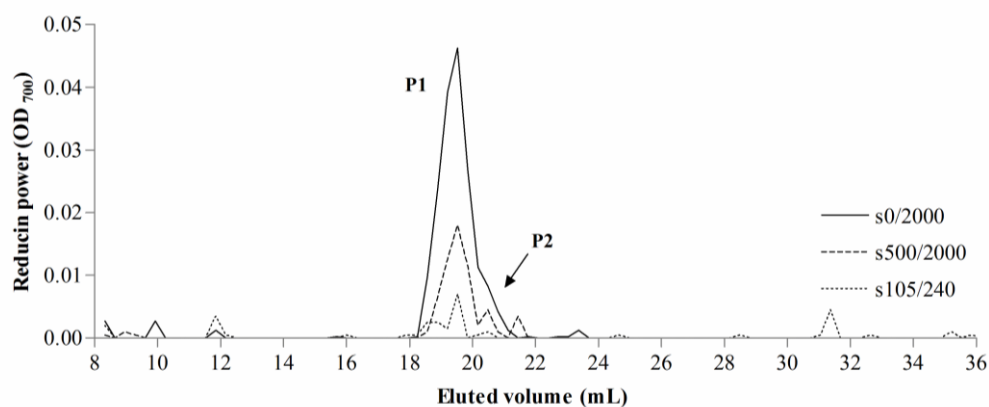


Figure 4.13 Changes in reducing power activity (OD_{700}) with nitrite and ascorbate addition in aqueous $\leq 10\text{kDa}$ extracts of model sausages s0/2000, s500/2000 and s105/240.

Although, the results did not provide unambiguous evidence to explain the relationships between the SEC chromatograms, added and residual nitrite/ascorbate and *in vitro* reducing power, the indications of a connection with ascorbate in particular is clear. However, it might be the case that the different methods detect different forms or maybe even reaction products of ascorbate.

In Paper II it was speculated that the activity in P2 was connected with phenylalanine or minor peptides. The steady appearance of the SEC peak II at day 0 in both model hams and sausages could indicate that the reducing power detected in this fraction was not connected with the additives and thus the phenylalanine/peptide hypothesis might be correct. However, the reducing power activity in P2 increased with time (Figure 4.14) indicating some active compound forming, potentially acting additively with the phenylalanine/peptide, during this period. Yet nothing final can be said on the nature of such a compound from the current data.

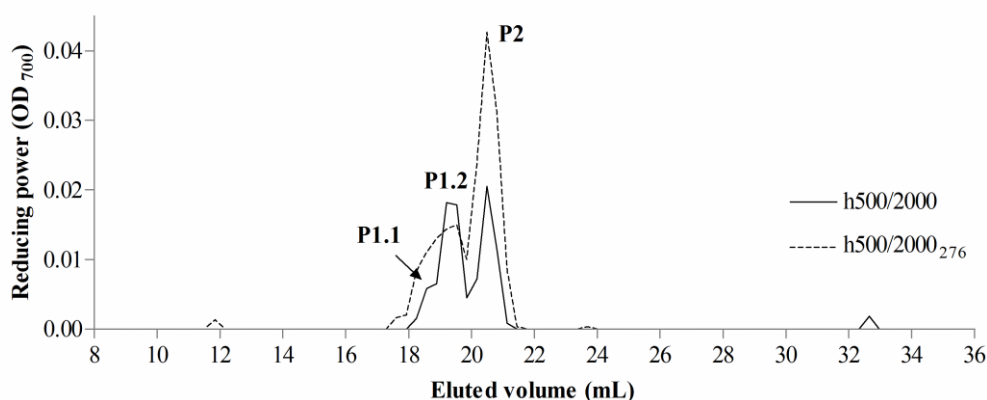


Figure 4.14 Changes in reducing power activity (OD_{700}) in peak-fractions P1 and P2 after cold storage of the model ham, h500/2000.

Just as was demonstrated for the fractionated commercial NCCPP extracts in Paper II ABTS radical scavenging activity in aqueous ≤ 10 kDa extracts of the model hams and sausages were divided into two peak-fractions either reacting fast (P3 – eluting at 22.1 mL) or slower (P4 – eluting at 19.21 mL) (Figure 4.15). P3 and P4 were present in all model aqueous ≤ 10 kDa extracts. In the sausages P3 was either equally as strong as P4 (after 31 min of incubation) or P3 was stronger than P4. In the model hams P4 tended to be stronger than P3 when not approximately equal. The SEC profile of the commercial NCCPPs did not follow a uniform pattern as for the model products. Otherwise no correlations were detected between ABTS radical scavenging activity and product type, nitrite/ascorbate addition or storage time for the model products. The association between SEC peak I and ascorbic acid discussed above did not transfer to the ABTS radical scavenging activity results, although P4 aligned with peak I and P1. This was evident from the 0/0 samples of model ham and sausages that both demonstrated ABTS radical scavenging activity in P4. Although, ascorbic acid is occasionally referred to as a radical scavenger it must be assumed that if ascorbate is present in the fraction in question, it does not scavenge the ABTS radical and thus the fraction must contain other antioxidative compounds such as peptides or free amino acids. This could for example be histidine or proline as was argued in Paper II. It is not to say whether such peptides might also have contributed to the reducing power activities.

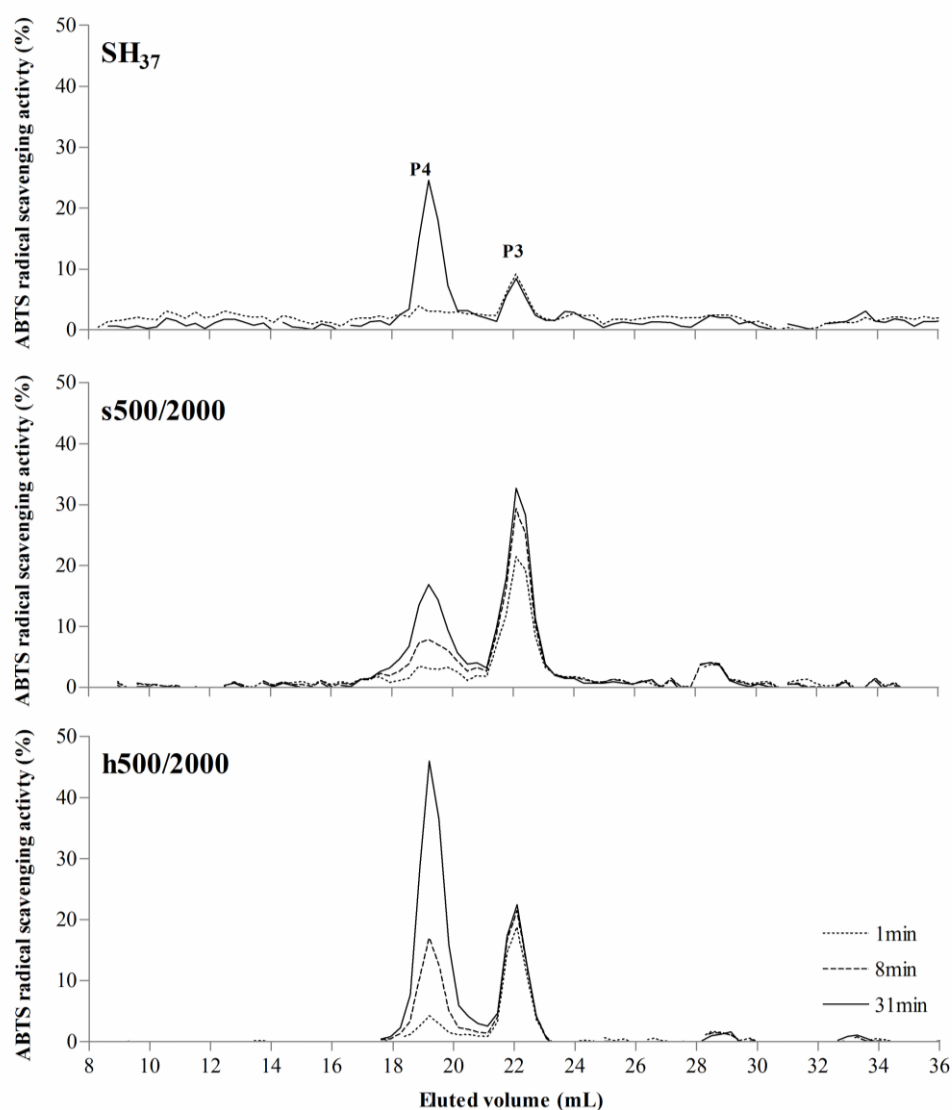


Figure 4.15 ABTS radical scavenging activity of SEC fraction of aqueous ≤ 10 kDa extract of the commercial restructured sandwich ham, SH₃₇, the model sausage, s500/2000 and the model ham, h500/2000.

To sum up, the results indicated that all types of NCCPPs (whole muscle, restructured and minced) does not contain compounds between 10 and 6.5 kDa in their aqueous low molecular weight fraction. Furthermore, this type of products resulted in similar SEC profiles that did, however, contain small differences related to the degree of comminution, additives and processing in general. The main correlations between the appearance of the SEC profiles and additives were that the 280/254 nm signals were affected by added ascorbate and decreased with time of cold storage.

Reducing power activity was found to be related to ascorbate yet the results were biased on product type and diluent in regard to the importance of added versus residual ascorbate/ascorbic acid. The indications of increasing reducing power with time presented in Paper III were also demonstrated in the current result. If

ascorbate is present in peak I/P1/P4 the chromatographic and colourimetric assays may not be responding similarly to the same form of ascorbate. It is, however, still likely that these fractions-peaks as well as P2 and P3 contain other antioxidant compounds that might, as argued in the papers, be of protein origin. This was confirmed as evidence pointed towards the reducing power in P2 originating from phenylalanine. Unlike, at previous occasions no correlation was found between nitrite/ascorbate and ABTS radical scavenging activity after fractionation.

It would be interesting to carry out the same characterisation changing the samples to comminute sausages with parallel increasing nitrite and ascorbate addition, and both hams and sausages of the full matrix experimental design described in Chapter 3.1.3 in order to further establish the importance of product type.

4.3 Part C – Antimicrobial activity

The following paragraph summarises the results from the investigations of antimicrobial activity otherwise presented in Paper I and Paper III and discusses potential further investigation strategies. All microbiological investigations were conducted by DMRI.

4.3.1 Studies of bacterial growth inhibition by extracts of commercial and model hams

The aqueous ≤ 10 kDa extracts of the three commercial NCCPPs as well as the model hams of parallel increasing levels of added nitrite and ascorbate were tested for their growth inhibitory properties against *L. monocytogenes* and *S. Typhimurium*. With the vast amount of literature in the field of inhibition of bacterial growth by nitrite (Buchanan & Solberg, 1972; Hustad *et al.*, 1973; Pichner, Hechelmann, Steinrueck, & Gareis, 2006 (Abstract); Reddy, Lancaster, & Cornforth, 1983) including inhibition of *L. monocytogenes* and *S. Typhimurium* (Hospital, Hierro, & Fernández, 2014; King *et al.*, 2016), the expectations were rather high and thus the aqueous ≤ 10 kDa extracts were tested in three different concentrations. However, none of the aqueous ≤ 10 kDa extracts of either commercial NCCPPs or model ham origin showed any antimicrobial properties, even at the highest tested concentration. Although, nitrite, active reaction product hereof and ascorbic acid (reported to, to some extent, inhibit microbiological growth of e.g. *L. monocytogenes* (Giannuzzi & Zaritzky, 1996; Valero, Carrasco, Pérez-Rodriguez, García-Gimeno, & Zurera, 2006)) were expected to be present in the low molecular weight aqueous fraction it was decided to investigate if an important antimicrobial agent had been excluded during the ≤ 10 kDa dialysis. Yet, testing the supernatants of the meat/water homogenates used for producing the aqueous ≤ 10 kDa extracts gave the same result. The absent antimicrobial response in the extracts but also in the homogenates may have been due to low test concentrations of the active component(s) as a consequence of the 1:15 dilution of the ham in connection

with extraction. Thus, strategies for concentrating the aqueous $\leq 10\text{kDa}$ extracts could be explored. When considering strategies for concentrating the samples, thoughts should be given to the consequences of potentially harmful actions e.g. applying heat (above the cooking temperature) and exposure to air. Optimizing the experimental design of the growth study focusing on limiting dilutions was considered, however, it was concluded not to be possible for the time being.

In addition it would be highly relevant to conduct a study comparing bacterial growth of a complete nitrite-cured cooked ham as well as the two aquatic fractions. Lastly, it would be interesting to see if the extracts are capable of inhibiting other microorganism such as the highly relevant *C. botulinum*.

4.4 Part D – Characterisation of constituent components in aqueous $\leq 10\text{kDa}$ NCCPP extracts

4.4.1 SDS-PAGE and biotin switch

One hypothesis central to the current PhD work is that in addition to the added preserving agents, nitrite and ascorbate, antioxidant and antimicrobial peptides play an important role in the extended shelf-life of cured meat products. Furthermore, that unique for nitrite-cured (cooked) meat products is antioxidant and antimicrobial activities performed by nitrite-induced modified meat constituent and more specifically nitrosated peptides.

As previously mentioned (Chapter 2.3.3) nitrosylmyoglobin and the somewhat smaller S-nitrosoglutathione and S-nitrosocysteine has been found to have antioxidative (and antimicrobial) properties (Chiueh & Rauhala, 1999; Incze *et al.*, 1974; Kanner, 1979; Møller & Skibsted, 2002). Common for the last two are that the nitrosation occurred on a thiol where the hydrogen has been substituted for NO – a so called S-nitrosation.

A method known as the biotin-switch assay was developed by Jaffrey, Erdjument-Bromage, Ferris, Tempst, & Snyder (2001) as a sulphur-based alternative to the many NO-based methods otherwise applied for the detection of S-nitrosated proteins (Forrester, Foster, Benhar, & Stamler, 2009). This method involves three sequential steps initiated by blockage of free thiols by MMTS followed by specific reduction of S-nitrosothiols to free thiols by ascorbate, and lastly labelling the newly generated thiols with biotin. Commercial assay kits build on this concept but slightly modified have been developed. By means of such kits the ambition was to perform an initial characterisation of the aqueous $\leq 10\text{kDa}$ NCCPP extracts proven to be antioxidative with focus on nitrosated peptides. By separating the extract components (post-biotin-switch) by SDS-PAGE followed by immunoblotting for detection of the biotin-labelled peptides we would get an impression of the extent and size of peptides in the aqueous $\leq 10\text{kDa}$ fraction to have undergone S-

nitrosation. After verification of the applicability of the method to the samples as well as the presence of nitrosothiols in the extracts, a deeper characterisation was to be conducted. The bands containing nitrosated peptides could then for instance be further analysed by MS. However, the intent was to obtain such a characterisation either by streptavidin pull down (Forrester *et al.*, 2009) or by carrying out purification by resin assisted-capture of SNO-proteins (SNO-RAC) (Thompson, Forrester, Moseley, & Foster, 2013) directly on the extracts, both of which was to be followed by identification by MS. It would then be possible to have the identified S-nitrosated peptides synthesised for individual analysis of *in vitro* antioxidant activities.

It was at the outset not possible to produce a SDS-PAGE with visible bands from the aqueous ≤ 10 kDa extracts of SH₀, DH₀ and SPS₀. Electrophoretic separation of small peptides is often problematic and despite several efforts to optimise the electrophoresis procedure the result remained. Di Luccia *et al.* (2015) found sarcoplasmic proteins of cooked hams and emulsion sausages to exhibit a very low extractability by a weak phosphate buffer. As these experiments (Di Luccia *et al.*, 2015) were also conducted on cooked cured pork products it was concluded that the lack of visible bands in the current situation had also to, at least in part, be due to low concentrations of extract protein caused by deep heat-induced conformational changes changing the proteins solubility compared to raw pork (Di Luccia *et al.*, 2015). As will be discussed in Chapter 4.5.1 the bicinchoninic acid (BCA) protein detection method applied in order to run comparable amounts of protein later turned out to be highly sensitive to residual amounts of added ascorbate. Thus, the actual protein concentrations loaded onto the initial electrophoresis gels were much lower than anticipated, confirming the suspicion of low protein content as part of the explanation for lacking bands.

As consequence of the initially blank SDS-PAGE it was decided to attempt visualisation of S-nitrosated proteins by means of a dot blot instead of the planned western blot. This would provide a yes/no answer for the presences of S-nitrosated proteins in the aqueous ≤ 10 kDa NCCPP extracts as well as give an opportunity to optimise the procedure to the samples prior to a potential purification by streptavidin pulldown or SNO-RAC. For this purpose an S-Nitrosylated protein detection kit from Cayman Chemical was acquired (item no. 10006518, Cayman Chemical). This kit functioned very similarly to the original biotin switch with blocking of free thiols utilising a compound undisclosed by manufacture, followed by reduction of –SNO and labelling by biotin. For the exact procedure see Chapter 3.3.3.

The kit was initially tested on freshly synthesised S-nitrosocysteine (in the following figures denoted CysNO). To accommodate any potential errors in this synthesis during testing of the kit, a cysteine sample was also introduced in the biotin switch after the blocking stage. The cysteine thiol groups would then be available for labelling just as any newly formed thiols reduced from –SNO. Both controls gave strong signals when developed with chemiluminescence (HRP conjugate with Amersham ECL Prime detection reagent kit, GE Healthcare) (Figure 4.16).

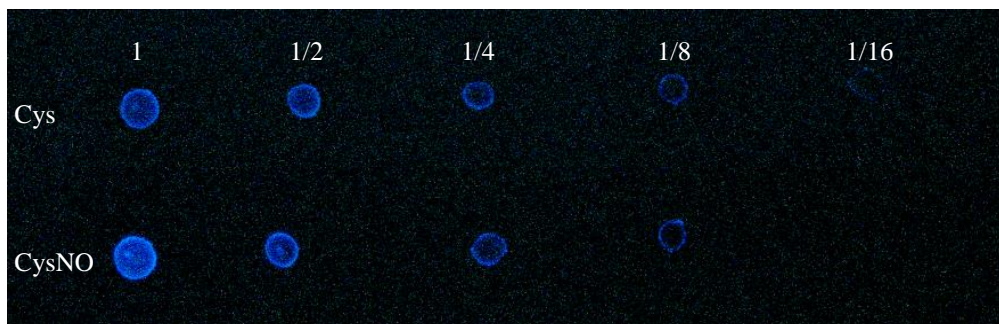


Figure 4.16 Dot blot of biotin-labelled S-nitrosocysteine (CysNO) and cysteine (Cys – was introduced into the biotin switch after blocking of free thiols). The samples were diluted before dotted onto the membrane. The concentration is halved for each dot towards the right.

The dot blot in Figure 4.16 indicated a semi-quantitative nature when the samples were diluted after the biotin switch. To confirm this, a small series of dilutions of freshly synthesised S-nitrosocysteine was subjected to the full biotin switch and then afterwards subjected to an additional dilution (Figure 4.17). The differences in the pre-biotin switch dilutions were not as clear as post-biotin switch dilutions, yet focusing on the more diluted samples they appeared to fade when moving towards the most diluted S-nitrosocysteine solution in the bottom right corner in Figure 4.17. Thus, it could be concluded that the biotin switch visualised by dot blot possessed semi-quantitative properties by comparison provided a concentration difference of a certain magnitude.

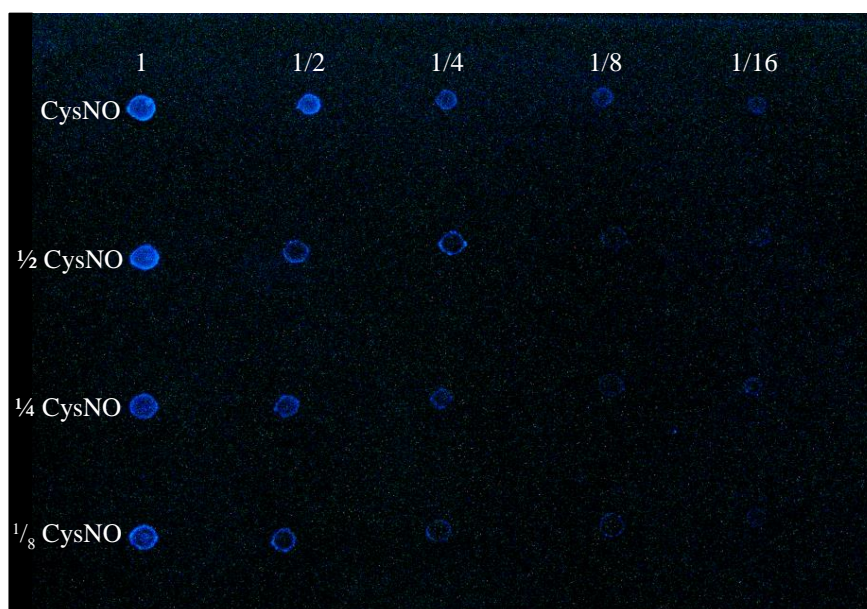


Figure 4.17 Dot blot of biotin-labelled S-nitrosocysteine (CysNO). Pre-biotin switch dilutions are indicated by the fraction before “CysNO”. Post-biotin switch dilutions before are indicated by the fraction in the top panel.

The dot plot of the labelled aqueous ≤ 10 kDa extract of the commercial NCCPPs (SH₀, DH₀, SPS₀) and the ham (h0/0) with no added nitrite/ascorbate used as reference (Figure 4.18) indicated that the extracts of the

three cured and the uncured ham contained S-nitrosated proteins. S-nitrosations are important for protein function under physiological conditions and although –SNO are unstable a basal level of S-nitrosated proteins could exist in meat (Bartberger *et al.*, 2001; Cammack *et al.*, 1999; Chen *et al.*, 2015). Furthermore, S-nitrosation has long been believed to occur in nitrite-cured meat and despite uncertainty of the presence of low molecular weight nitrosothiols (Gilbert, Knowles, & McWeeny, 1975; Sullivan & Sebranek, 2012), nitrosothiols have been detected on several occasions (Cassens *et al.*, 1979; Emi-Miwa, Okitani, & Fujimaki, 1976). Although, it was expected for aqueous $\leq 10\text{kDa}$ extracts of the cured products to contain more S-nitrosated proteins than the uncured reference no concentration difference was evident from the blot of the crude aqueous $\leq 10\text{kDa}$ extracts. When a dilution similar to the one applied to S-nitrosocysteine (Figure 4.17) was performed on the aqueous $\leq 10\text{kDa}$ extract of SH_0 fading also took place with pre- and post-biotin switch dilution (data not shown). Thus, the semi-quantitative properties of the biotin switch seen on S-nitrosocysteine also applied to the samples meaning that the results in Figure 4.18 indicated that the presence of S-nitrosated proteins in aqueous $\leq 10\text{kDa}$ ham extracts did not correlate with added nitrite.

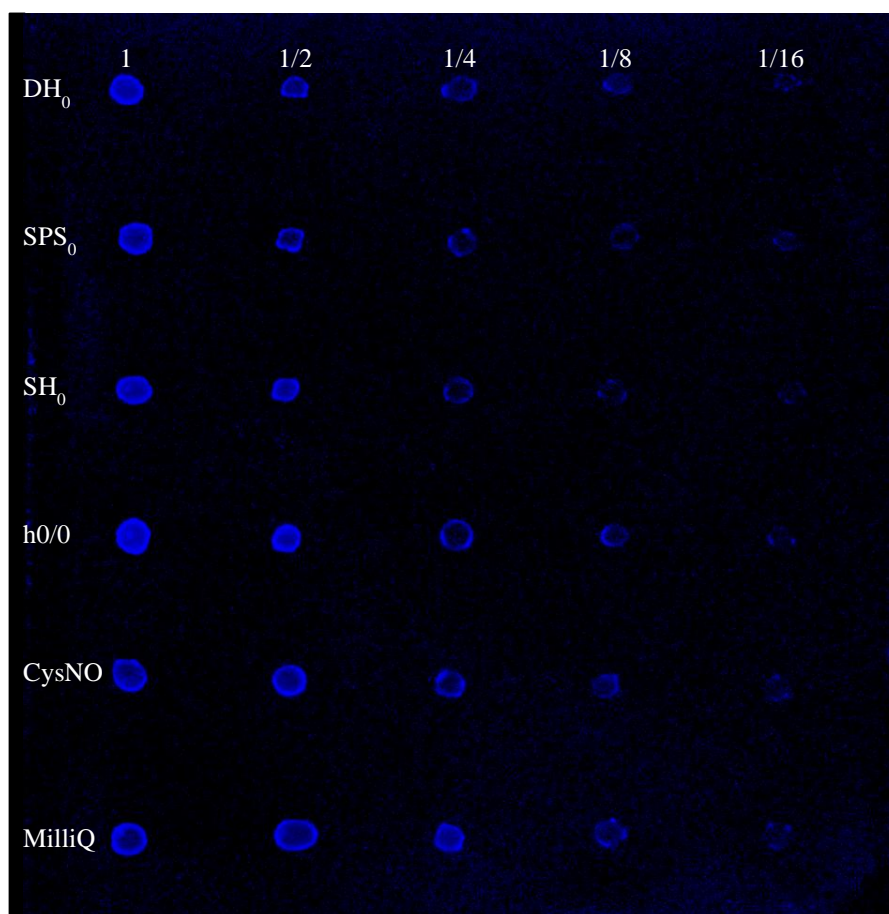


Figure 4.18 Dot blot of aqueous $\leq 10\text{kDa}$ extracts of DH_0 , SPS_0 , SH_0 and h0/0 as well as S-nitrosocysteine (CysNO) and MilliQ water subjected to biotin labelling by the biotin switch. Post-biotin switch dilutions before are indicated by the fraction in the top panel.

However, in the dot blot in Figure 4.18 a clear signal was also detected from the MilliQ water used as negative control in the biotin switch. Suspicions of a potential contamination of the MilliQ water was dismissed as the same result occurred on repetition of the experiment with fresh MilliQ water as well as dH₂O (data not shown). In order to establish the origin of the water signal, blot development by means of a fluorescein-conjugated streptavidin was also attempted, yet the signal remained. Thus, it was established that the error was most likely associated with the labelling procedure and potentially due to insufficient removal of excess biotin. This theory is slightly weakened as it is considered unlikely that water, with no binding sites, would retain as much biotin as protein containing samples and thus, we would at least expect some signal differences as was the case for samples and controls of different concentrations. Chen *et al.* (2015) applied the same nitrosylation kit from Cayman Chemical on brain tissue and was able to produce a nice differentiable dot blot. However, they reported nothing on the use of negative controls. Hence, this offers no information on whether a signal in the negative control is a common problem. To my knowledge no other data has been published on this problem.

In order to circumvent the challenges with the negative control an alternative S-nitrosation detection kit was tested. The Pierce™ S-Nitrosylation Western Blot Kit from Thermo Fischer Scientific also applies the biotin switch principles, yet labels with iodoTMT instead of biotin and detects by means of an antiTMT antibody and a secondary goat anti-mouse IgG-HRP antibody. Furthermore, they recommend using reduced glutathione and S-nitrosoglutathione as negative and positive control, respectively. For the exact procedure see Chapter 3.3.3. However, the experiment was unsuccessful and resulted in a blank membrane for all samples and control.

As it was not possible to produce a trustworthy result with the applied methods, it was considered too high-risk to continue with an experimental strategy build on the biotin switch. However, even though it was not possible to provide evidence for the presence of S-nitrosated proteins in aqueous ≤ 10 kDa extracts of commercial NCCPPs with the current experimental set up, it cannot be ruled out that such modified proteins are in fact formed during nitrite-curing.

The SDS-PAGE of a NCCPP (meat/water homogenate) produced on much later occasion showed visible bands in the water-soluble fraction (see Appendix A). The gel clearly emphasised that the NCCPPs contained a very low concentration of water soluble proteins. Thus, the aqueous ≤ 10 kDa fraction constituting the foundation of this PhD study would be expected to contain even less protein capable of giving rise to an electrophoretic protein pattern. As the result presented above did not give an impression of degree of sample S-nitrosation, it is not possible to predict whether the limited amount of protein in the aqueous ≤ 10 kDa extracts would be sufficient for detecting S-nitrosated proteins by immunoblotting combined with the biotin switch after electrophoretic separation.

Di Luccia *et al.* (2015) reported their myofibrillar protein extract to demonstrate an unexpected complex electrophoretic protein pattern showing sarcoplasmic protein bands that co-migrated with several myofibrillar components. The unusual composition of this fraction combined with the large amounts of protein detected in the remaining fraction of meat/water homogenate (Appendix A) would make it highly interesting to further investigate the fraction not soluble in water in regard to S-nitrosations.

4.3.2 Mass Spectrometry (MS)

As –SNO could not be detected by the biotin switch method an attempt to purify by streptavidin pulldown or SNO-RAC was cancelled. Instead other approaches for characterisation of the antioxidative aqueous ≤ 10 kDa NCCPP extracts were considered.

A bottom-up proteomic technique commonly referred to as shotgun was applied to a SEC separated aqueous ≤ 10 kDa extract sample in a collaboration between Aalborg and Aarhus University. This technique generally comprises enzymatic digestion of a complex protein mixture followed by separation and identification of the resulting peptides, using a combination of LC and MS (Zhang, Fonslow, Shan, Baek, & Yates, 2013). Another, MS-based analysis strategy aiming at providing a broader picture and thus not confined to focusing on protein was also attempted. Here, in addition to the crude aqueous ≤ 10 kDa NCCPP extracts, a ≤ 3 kDa fractionation generated by UF (Vivaspin6 3000Da MWCO centrifugal concentrators – Sartorius) were also analysed. These were then attempted analysed by direct flow injection and LC-MS on an ESI-QTOF instrument. For details on the procedure see Chapter 3.3.4. Lastly, LC-MS were also applied in the search for a specific modification (C-nitration) on proteins potentially occurring as a consequence of nitrite-curing (Feng *et al.*, 2016). Thus, a method for the detection of 3NT was developed in collaboration with University of Copenhagen (Paper I).

Shotgun analysis (conducted by Aarhus and Aalborg University)

Shotgun analysis of selected SEC fractions (corresponding to SEC peak I, II, III and IV in Figure 4.19) of the aqueous ≤ 10 kDa SH₀ extract revealed the extract to be a very complex mixture of peptides/proteins. Although, the total protein content was low, the aqueous ≤ 10 kDa extract contained peptides originating from 95 different proteins of pig origin across the tested SEC fractions (Appendix B). Even though, all the SEC fractions contained peptides determined to be from the same six proteins, it was not the same peptides found in all the SEC fractions.

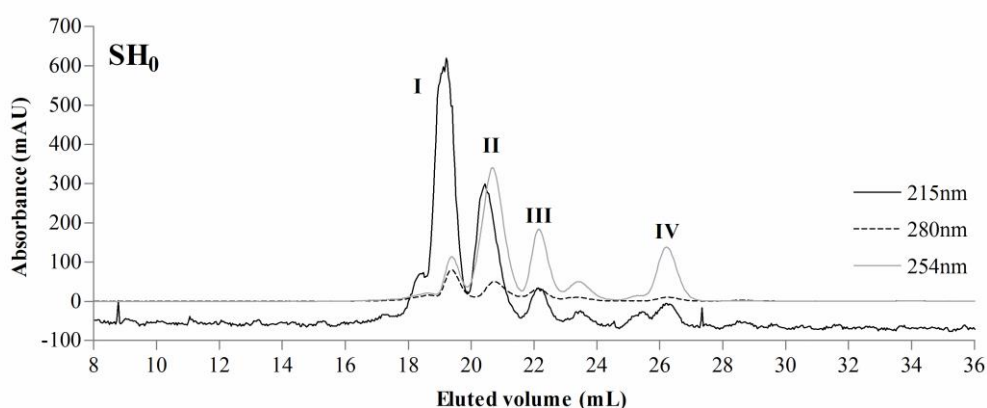


Figure 4.19 Representative SEC chromatograms of the aqueous $\leq 10\text{kDa}$ peptide extract of SH_0 .

The majority of the identified proteins/peptides were non-structural muscular proteins. However, peptides of actin, tropomyosin and myosin light chain were also identified in the extract SEC fractions. With the exception of one actin unique peptide in fractions from SEC peak II and III the myofibrillar peptides were compiled in fractions from SEC peak I. SEC peak I has on previous occasions been found to coincide with *in vitro* ABTS radical scavenging activity (slow acting) and weak reducing power activities (P4 og P1 in Figure 4.3 in Chapter 4.1.1). Antioxidant activity detected in fractions corresponding to SEC peak I was in Paper II argued to be of peptide/protein origin. Thus, it can be hypothesised that these muscular peptides fragments may have contributed to the detected antioxidant activity unique to SEC peak I. It is not possible to establish whether these peptides are the result of protein breakdown during ham processing or related to common cell household breakdown. Neither can it be verified whether potentially antioxidative muscular peptides may be connected with nitrite-curing, as the shotgun analysis was only set up to collect data for a basic characterisation of constituent proteins and not to look for nitrite-induced modifications e.g. nitrosations and nitrations.

LC-(ESI)-MS analysis (conducted at the National Food Institute)

The LC-(ESI)-MS analysis was intended to characterise the aqueous $\leq 10\text{kDa}$ extracts in an even broader perspective in order to also get an impression of the content of non-protein constituent that might have contributed to the detected antioxidant activity. The MS analyses was carried out in three different experimental set ups. The set ups were (I) direct flow injection of diluted $\leq 3\text{kDa}$ extract fractions (II) separation on a C8 column followed by MS analysis in m/z 300-2900 on aqueous $\leq 10\text{kDa}$ extracts, $\leq 3\text{kDa}$ extract fractions, and concentrate from the UF and (III) separation on a C18 column followed by MS analysis

in m/z 50-1000 on aqueous ≤ 10 kDa extracts of SPS_0 and $h0/0$ (for details see Chapter 3.3.4). MS/MS fragmentation was performed in connection with set up III.

No peaks were detected using flow injection (data not shown). Thus, to acquire any information about the samples it was necessary to separate and retain the sample components in the system a little longer and for this purpose a C8 column was mounted onto the system (set up II). Analysis of the crude aqueous ≤ 10 kDa NCCPP extracts, ≤ 3 kDa fractions and extract concentrates (above 3kDa MWCO UF filter) resulted in more or less identical peak profiles for all three sample categories. The lack of difference between different extract fractions may have been due to erroneous fragmentation during the UF or that the extract simply did not contain compound larger than 3kDa. Fast protein liquid chromatography analysis by SEC of the extracts had on previous occasion revealed the extract to only contain components smaller than 6.5kDa (Figure 4.2 and Figure 2 in Paper II). As no size marker between 6.5kDa and 200Da was subjected to SEC, it cannot be ruled out that the extract constituents were not just below 6.5kDa but in fact below 3kDa in size.

Overall the base peak chromatograms (BPCs), from the initial LC-MS analysis (set up II), looked relatively similar for the commercial nitrite-cured samples and the uncured reference, separating the sample constituents in 7-12 peaks (Figure 4.20). These peaks were generally present in all samples but with varying signal intensity. In experimental set up II, five main peaks in the BPC were further investigated (marked on Figure 4.20). Four of these peaks were clearly present in all the tested aqueous ≤ 10 kDa ham extracts (BPC peak number 1, 2, 3, 5). The MS spectrums of BPC peak 1 indicated a potential tetramer and BPC peak 3 indicated a potential dimer while BPC peak 5 was dominated by adducts (data not shown). Nothing could be deduced from the MS pattern of BPC peak 2 with the information at hand. BPC peak 4 was very clear in SPS_0 yet was also present in $h0/0$ and DH_0 . The MS spectrum of BPC 4 only contained one ion namely m/z 861.

No ion was, based on the current analysis, only detected in all the hams added nitrite. The restructured sandwich ham, SH_0 was the one standing out by not containing the 861 ion detected in both experimental set up II and III. By further analysis and fragmentation (set up III), the 861 ion was established to be dimer of a 430 component (Figure 4.21) – any smaller fragments were identified as adducts.

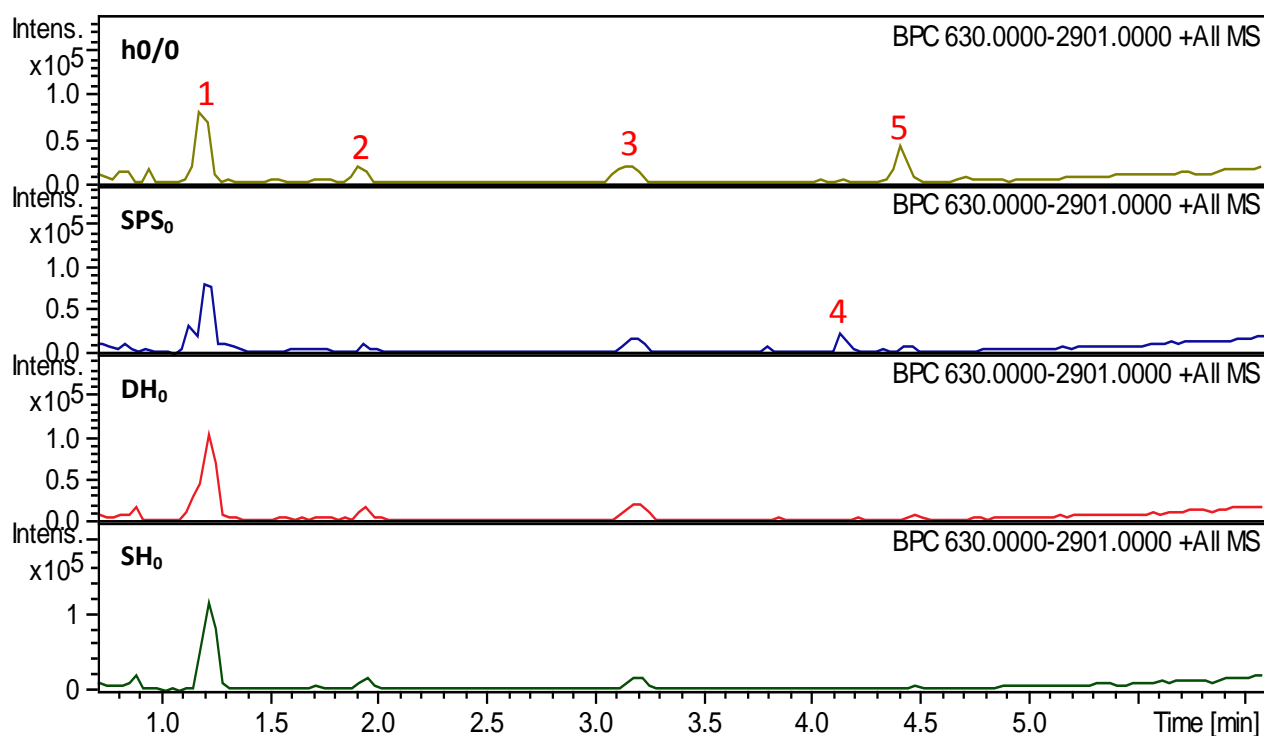


Figure 4.20 BPC of $\leq 3\text{kDa}$ fractions of the aqueous $\leq 10\text{kDa}$ ham extracts from the reference ham, h0/0 and the commercial NCCPPs SPS₀, DH₀ and SH₀. The BPC originates from the method with m/z range 300-2900 – however, for this illustration only m/z 630-2900 is presented.

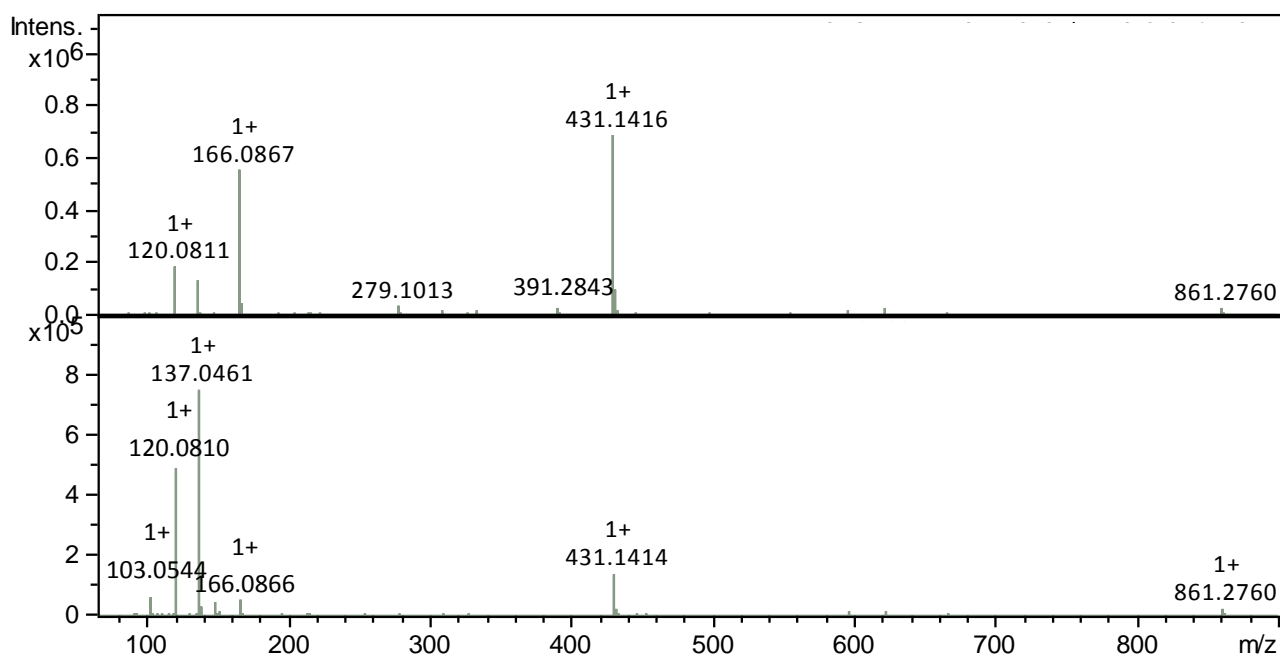


Figure 4.21 Comparison of mass spectrum of a base peak (in BPC from the aqueous $\leq 10\text{kDa}$ extract of SPS₀ in set up II) found to contain ion 861 (top) with the mass spectrum of the fragmentation of ion 861.2760 (bottom).

Despite the use of different analysis strategies including MS/MS fragmentation, different search engines and a sample comparison taken notice of signal differences above a certain factor threshold, it was not possible to identify many of extract compounds. The 431 ion were identified as either ononin or formononetin (isoflavonoids potentially originating from feed - ononin from e.g. soybeans and formononetin from clover or legumes in general (Budryn *et al.*, 2018; Hloucalová *et al.*, 2016)) with the most evidence pointing to ononin. Another ion (m/z 177) discovered by the comparison of h0/0 and SPS₀ (data from set up III) was identified as ascorbic acid. As ascorbic acid (in the form of sodium ascorbate) was added to SPS₀ in connection with nitrite-curing and SPS₀ was the commercial NCCPP found to contain the highest concentration of residual ascorbic acid (Table 3.2 in Chapter 3.1.1), the current detection of its presence in much higher concentrations in SPS₀ vs h0/0 is considered correct.

The aqueous ≤ 10 kDa extracts had previously been established to contain a wide variety of peptides yet the current method was not developed to look selectively for peptides. In spite of this one noticeable ion, m/z 395.1558, was matched to a tri- and tetra-peptide. The tripeptides consisted of aspartic acid, phenylalanine and asparagine in different order while the tetra peptides were composed of one aspartic acid, one phenylalanine and two glycines, also in varying order. The presence of most of these amino acids in a peptide have been associated with antioxidant properties (Carrasco-Castilla *et al.*, 2012; Marcuse, 1962). However, phenylalanine did not demonstrate high reducing power and ABTS radical scavenging activity when tested as free amino acid in solution (see Chapter 4.5.2). Thus, it is difficult to establish the importance of such. The remaining ions and their calculated gross formulas resulted in an immense list of potential compound matches. These were generally complex structures often containing very condensed ring structures. The result did not in any way point towards S-nitrosated peptides. However, a selection of these hits contained buzz elements as pyrimidine, adenosine, purine as well as nitro in the systematic names (Appendix C). Although, nitric oxide and related compounds have been found to interact with and modify DNA, the resulting reaction products may to the best of my abilities be concluded to be far from the current compound hits (Burney, Caulfield, Niles, Wishnok, & Tannenbaum, 1999). It has been established that nitroso compounds from faecal matter after ingestion of nitrite-cured meat are capable of inducing *in vitro* DNA damage (Joosen *et al.*, 2009). Yet to my knowledge there are no published studies on NO-dependent DNA modifications occurring post-mortem in nitrite-cured meat. However, if such modifications in fact do occur, the reaction products would be expected to be similar to some of the ones found *in vivo* and *in vitro*.

Although, some information could be drawn from the MS analyses applied with the available resources, further and more advanced investigation are required in order to fully characterise the aqueous ≤ 10 kDa extracts which is considered highly valuable for a full understanding of the antioxidative activities occurring in NCCPPs

3-nitrotyrosine on LC- MS (conducted in collaboration with University of Copenhagen – Paper I)

As the previously applied narrow biotin switch and broader MS analyses described above were unsuccessful in identifying nitrite related modification on proteins and peptides, an alternate approach was considered. Sodium nitrite is, as has already been mentioned, generally regarded as an inhibitor of lipid oxidation. However, its influence in regard to protein oxidation has not revealed a significant antioxidant effects. In addition to ROS, RNS may also be present in meat systems subjected to nitrite-curing (Skibsted, 2011), why initiation of both oxidative and nitrosative reactions may be expected to occur. However, the manifestation of such reactions and the connection between oxidative and nitrosative reactions as well as their consequences on cured meat products are not fully elucidated. Exposure of tyrosine to RNS such as nitrogen dioxide and in particular peroxynitrite, leads to the formation of 3NT (Villaverde, Parra, *et al.*, 2014; Vossen & De Smet, 2015) which has long been used as a marker for nitrosative stress *in vivo*. Yet, 3NT has also been found in meat products (Feng *et al.*, 2016; Vossen & De Smet, 2015) and has furthermore been found to increase with nitrite addition (Villaverde, Morcuende, *et al.*, 2014). Due to the potential interlink between oxidative and nitrosative reactions, 3NT has been suggested as a potential marker for protein oxidation in raw and cooked nitrite treated meat (Feng *et al.*, 2015). Thus, investigating 3NT may give an impression of the role of nitrite-induced peptide modifications in the redox state from another angle than as active antioxidant compounds

In the current investigation of the presences of 3NT in the three commercial NCCPPs (SH₀, DH₀, SPS₀) and the reference with no added nitrite/ascorbate (h0/0), 3NT was found in neither the ≤10kDa fraction nor the meat. If 3NT can in fact be considered a proper marker for protein oxidation the current results would indicate limited protein oxidation potentially due to the presence of strong antioxidant compounds.

With the applied method detecting 3NT standard solution down to 0.5ng/mL it is odd that no 3NT was detected at all, considering that a basal level of 3NT, most likely originating from physiological nitrosative stress, is usually considered to be present in any muscle-based food (Villaverde, Parra, *et al.*, 2014). The absence of detectable 3NT may be partly explained by the addition of ascorbate as well as heat treatment as both have been found to cause a decrease in 3NT (Feng *et al.*, 2015; Villaverde, Parra, *et al.*, 2014).

3NT spiking of the aqueous ≤10kDa extracts led to a recovery of approximately 80%. However, no 3NT was recovered from spiking of the meat samples. This could indicate that 3NT formation may not just be inhibited in the complete NCCPPs, it might even be degraded, and that the degradation reaction(s) only occurs in the presences of unknown compounds >10kDa. Crow & Beckman (1995) pointed out that nitro-tyrosine may be reduce to amino-tyrosine under the conditions of an acid hydrolysis and furthermore indicated that such a reduction may be caused by thiol reductants. They also found that the conversion was generally higher when nitro-tyrosine was added to a complex protein mixture prior to hydrolysis indicating the mixture to contain compounds inducing the reduction (Crow & Beckman, 1995). Thus, it can be

speculated if such reactions may explain, at least in part, the lack of signal in the spiked meat compared to the spiked aqueous ≤ 10 kDa extracts.

4.5 Part E – Ascorbic acid and free amino acids in colourimetric *in vitro* assays

4.5.1 BCA protein assay and ascorbic acid in NCCPPs

Throughout the experimental work of this PhD study protein content was to be determined in a vast amount of liquid samples. The resistance to non-ionic detergents and buffers commonly used (Smith *et al.*, 1985), the one-step simplicity and commercial availability of the BCA method made this the first obvious candidate of choice. Furthermore, unlike the dye-based methods e.g. the Bradford method, the peptide backbone also contributes to colour formation in the BCA method, thereby helping to minimise variability caused by protein compositional differences. The BCA protein assay is built on a two-step concept depending on the biuret reaction. First, peptides containing three or more amino acid residues form a complex with Cu^{2+} in an alkaline environment (Smith *et al.*, 1985). During the biuret reaction Cu^{2+} is reduced to Cu^{1+} which is the cause of the light blue colour formation. The biuret reaction in itself constitutes a simple colorimetric protein detection method, yet the sensitivity is too low for most research purposes. In the second step of the BCA method, BCA chelates the newly formed Cu^{1+} effectively releasing the weakly chelated peptides. The $\text{BCA}/\text{Cu}^{1+}$ complex has an intense purple colour with a strong absorbance at 562 nm. Furthermore, it is much more sensitive than the biuret reagent (Smith *et al.*, 1985).

Using the PierceTM BCA Protein Assay Kit (Thermo Fisher Scientific (Thermo Fisher Scientific, n.d.-c)) the aqueous ≤ 10 kDa extract of the commercial NCCPPs cold-stored for 0 and 37 days were found to have a protein content of 0.2-0.6 mg/mL. The absence of a considerable change with time of cold-storage and by comparing to Kjeldahl protein in the hams (Table 3.2 – Chapter 3.1.1) the BCA determined protein concentrations were considered plausible. Differences in protein content between the aqueous ≤ 10 kDa extracts were then merely ascribed to the different nature and basic composition (fat, protein and water) of the three commercial NCCPPs (Table 3.2 – Chapter 3.1.1) as well as the aqueous ≤ 10 kDa extracts being made from slightly different amounts of ham. However, applying the BCA method to the aqueous ≤ 10 kDa extracts from the model hams saw doubts about the results.

BCA protein determination on the aqueous ≤ 10 kDa extracts from the model hams analysed in Paper III are presented in Figure 4.22. The model hams were produced by exactly the same recipe, so the extent of variation in protein content between the extract samples was much unexpected and thus believed to be an error yet reproducing the analysis gave the same result. By the process of elimination either nitrite or

ascorbate were expected of interfering with the assays as these were the only two variables in the model hams. Although, robust to many detergents and buffers used in protein contexts the BCA method is, unfortunately, sensitive to anything capable of reducing copper ions in an alkaline medium. The reducing nature of ascorbate could therefore cause it to reduce Cu^{2+} to Cu^{1+} and thus lead to more colour formation, affecting the accuracy of the protein quantification.

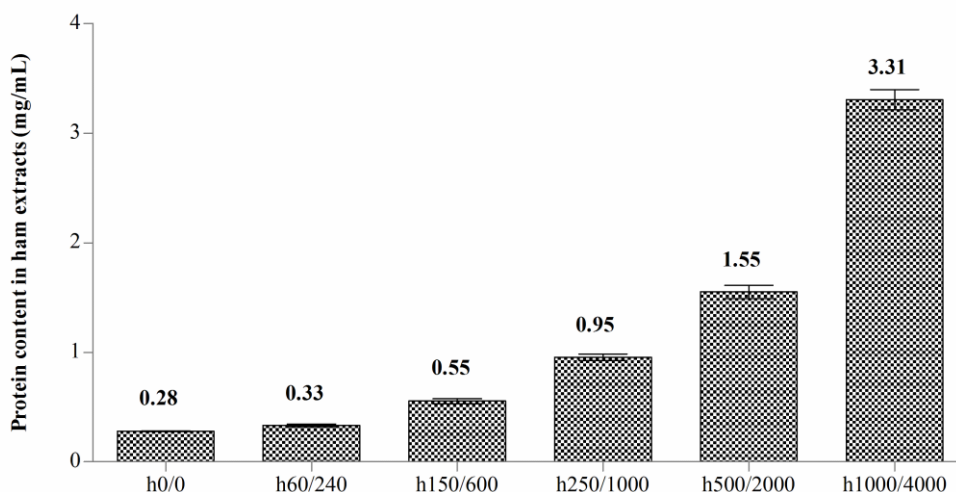


Figure 4.22 Protein content in aqueous $\leq 10\text{kDa}$ extracts of model hams of varying nitrite and ascorbate addition determined using the BCA method.

The acidic counterpart of ascorbate salts, ascorbic acid, has previously been reported to affect the BCA method even when present in small amounts (Jampel, 1994; Marshall & Williams, 1992; Slocum & Deupree, 1991; Williams *et al.*, 2003). This information was also indicated by manufactures telling to avoid even minute amounts of ascorbic acid. Thus, solutions of ascorbic acid in concentrations spanning the range of residual ascorbic acid detected in all aqueous $\leq 10\text{kDa}$ NCCPP extracts (as well as content converted from hams) were assayed using the BCA kit to confirm whether ascorbate/ascorbic acid could in fact lead to colour formation in the relevant concentrations (Figure 4.23).

The concentration of ascorbic acid solutions demonstrated a linear relationship with detected absorption/colour formation (Figure 4.23). By means of a standard curve produced from applying the PierceTM BCA Protein Assay Kit to solutions of BSA supplied with the kit (calibration curve: $130.62x^2 + 560.97x$) the BCA colour signal from the $100\mu\text{g/mL}$ ascorbic acid solution corresponded to a protein concentration of 1.4mg of protein per mL samples which equals to an extra contribution of 500% compared to the protein content in the h0/0 extract sample. Just as it was observed in the current study Williams *et al.* (2003) reported ascorbic acid to produce a linear and non-turbid response in the absence of protein. They also found the interference of ascorbic acid to occur in the presence of protein (only BSA). However, it was

less than additive. It would seem that the ascorbic acid and BSA were somehow interacting/competing in the biuret reaction. In order to test for any matrix effects in the more complex matrix that the aqueous ≤ 10 kDa NCCPP extracts represents the h0/0 sample were spiked to contain 0-50 $\mu\text{g/mL}$ ascorbic acid. The absorbance measured in an ascorbic acid solution was significantly smaller ($P < 0.05$) than the absorbance of a spiked sample of the same ascorbic acid spiking concentration yet the difference was smaller than the contribution from the h0/0 sample alone. Thus, the interference could, as for Williams *et al.* (2003), not be said to be strictly additive.

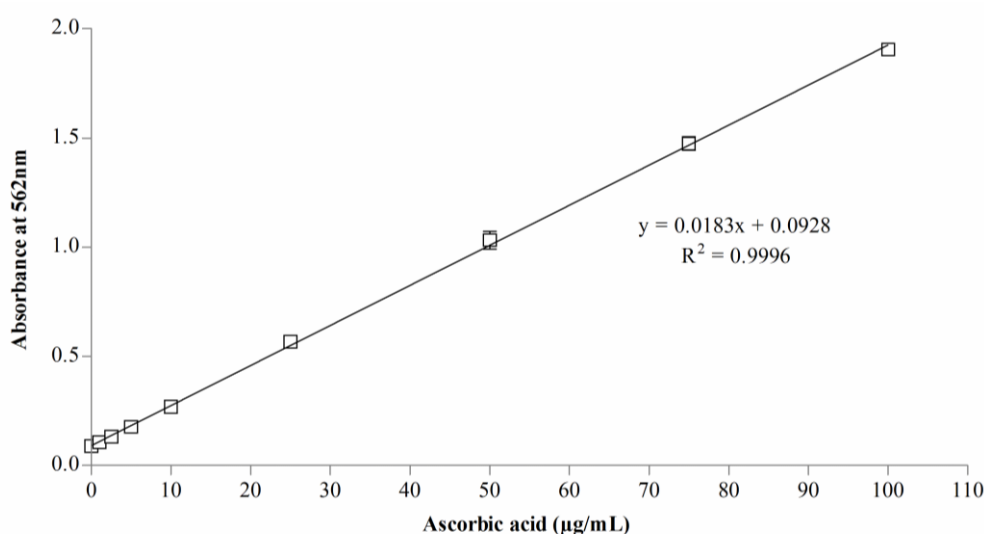


Figure 4.23 Absorbance detected at 562nm from ascorbic acid solutions reacting in the BCA method.

Attempts on finding strategies to circumvent the interferences from compounds like ascorbic acid on the BCA method have been published (Shihabi & Dyers, 1988). However, the benefits of such optimisations were not considered solid enough to match the workload required to attempt to implement such optimisations for the current situation. Thus, an alternative protein assay would have to be considered for samples expected to contain ascorbic acid. Although, the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951) is also copper based and functions by means of biuret reaction, the Modified Lowry Protein Assay Kit (Thermo Fisher Scientific (Thermo Fisher Scientific, n.d.-a)) are less sensitive to ascorbic acid and is listed by the manufacture as having a compatible substance concentrations of 1mM towards ascorbic acid (Jampel, 1994; Thermo Fisher Scientific, n.d.-b; Williams *et al.*, 2003). To verify its applicability for our samples the same pure ascorbic acids solutions and the spiked h0/0 samples as tested above were subjected to the Modified Lowry Protein Assay Kit. Fortunately, no effect of ascorbic acid was evident from the analysis. Using the Modified Lowry kit on all sample extracts mentioned above gave a protein concentration across all model and commercial NCCPP ≤ 10 kDa extracts of $0.2 \pm 0.03 \text{ mg/mL}$, and there was no apparent systematic variation

related to added ascorbic acid. This level was also consistent with the levels found in the aqueous ≤ 10 kDa extract of h0/0 when using the BCA kit.

Though, the BCA method was deemed non-compatible for determining protein content in aqueous NCCPP extracts high in ascorbic acid, the linearity of the colour development inspired the idea, that BCA could be used to estimate ascorbic acid in this type of samples. A BCA-based method for determining ascorbic acid in aqueous humor was developed by Shichi, Page, Sahouri, & Shin (1997). The referred method builds on two key principles (I) eliminating protein-induced absorbance signal by TCA precipitation prior to BCA reaction and (II) determining the ascorbic acid content by the absorbance difference between a sample where ascorbic acid had been oxidised and an untreated sample. In order to apply this it would be necessary to first examine if a TCA precipitation would be sufficient to rid the aqueous ≤ 10 kDa NCCPP extracts of all protein/peptide-induced signal that might interfere. For example, very small peptides will not precipitate but may contribute to the BCA colour formation. Furthermore, the amino acids cysteine, cystine, tryptophan and tyrosine (Wiechelmann, Braun, & Fitzpatrick, 1988) are together with the peptide backbone greatly contributing to the BCA colour formation. Hence, these four amino acids as free amino acids, if present, will not precipitate and may thus contribute to the signal. Shichi *et al.* (1997) rejects the importance of other known BCA method interferences (uric acid, glucose and glutathione) by proving that they are not oxidised like ascorbic acid under alkaline treatment. Any other reducing compounds present in the NCCPP extracts would at least have to be tested in the same manor. After these minor investigations this method could well pose a good starting point for the development of a simple ascorbic acid detection method for NCCPP extracts with lower sensitivity but higher throughput compared to the HPLC method described in Chapter 3.2.5.

4.5.2 In vitro reducing power and ABTS radical scavenging antioxidant activity of free amino acids

A contribution from free amino acids (in addition to small peptides and non-peptidic compounds) to the antioxidant activities measured in the low molecular weight aqueous NCCPP extracts was considered reasonable in the current situation. Thus, it was desirable to be able to evaluate the contribution from free amino acids in the applied assays (before and after SEC fractionation). Accordingly, 10 amino acids, either previously reported to possess antioxidant properties (as free or embedded in peptides) or present in high concentrations in the extracts, were selected for analysis. The 10 selected were histidine, methionine, phenylalanine, tryptophan, cysteine, tyrosine, proline, glutamic acid, glycine, and alanine. The concentrations of the free amino acid under investigation were adapted to the content determined in the aqueous ≤ 10 kDa NCCPP extracts (Paper II – Figure 4). Higher concentrations, up to 1mg/mL (the stock used for making the remaining concentrations), were also tested for three reasons (I) in the matter at hand to verify if a potentially absent antioxidant activity signal could merely be due to low concentration but also (II)

to evaluate if the concentration dependency on the interconversion between anti and pro-oxidant activities previously reported (Marcuse, 1962) is relevant in the current experimental set up and (III) on a general basis to investigate whether the antioxidant activities of free amino acids previously reported in the literature (by means of many different methods) could be confirmed using the current colourimetric *in vitro* assays.

ABTS radical scavenging was measure after incubating for 1, 8 and 31min at room temperature to be able to follow any kinetic differences between the different amino acids and solvents (Figure 4.24). Solutions of cysteine, tryptophan and tyrosine showed the strongest ABTS radical scavenging activity and at 0.05mg/mL these amino acids had reached the assay maximal capacity of approximately 93%. This maximum was reached already within the first minute of incubation. At the lowest tested concentrations the decreasing order of ABTS radical scavenging activity was cysteine (0.005mg/mL) > tryptophan (0.002mg/mL) > tyrosine (0.002mg/mL). The magnitude of the activity increased with incubation time, but the internal order remained. Furthermore, at this low concentration the activity was higher when the amino acid was dissolved in water, compared to ammonium acetate. For cysteine and tryptophan the activity detected in the two solvents was significantly different at all time points. The tyrosine solutions were turbid due to improper solubilisation in the 1mg/mL stock solution. Any precipitate would result in an increased absorbance leading to a decrease in the calculated colour reduction (ABTS radical scavenging activity). However, the turbidity became less pronounced as the stock was diluted to the tested concentrations. Furthermore, as the strong tyrosine solutions were capable of a full colour reduction (assay max) comparable to cysteine and tryptophan, the turbidity is not considered problematic for the overall conclusion of tyrosine exhibiting ABTS radical scavenging activity.

Antioxidant activity from solutions of tryptophan was established decades ago in systems of linoleic acid and its methyl esters (Marcuse, 1962). Over time solution of tryptophan as well as tyrosine were also more specifically found to act as radical scavengers in different *in vitro* assays e.g. ORAC-FL and an ABTS-based assay different from the one applied in the current work (Hernández-Ledesma, Dávalos, Bartolomé, & Amigo, 2005; Meucci & Mele, 1997). More recently, free tryptophan and tyrosine isolated from extracts of egg yolk was also found to act as strong ABTS radical scavengers (Nimalaratne, Lopes-Lutz, Schieber, & Wu, 2011). This scavenging ability of the aromatic tyrosine and tryptophan is believed to be connected to the phenolic and indolylic groups, respectively, enabling these amino acids to act as proton/hydrogen donors to electron deficient radicals and efficiently scavenge them. Also the aromatic structure of tyrosine is considered a major contributor to its antioxidant activity (Carrasco-Castilla *et al.*, 2012; Hernández-Ledesma *et al.*, 2005). The antioxidant capacity of tyrosine and tryptophan are most often reported to be very similar as was also evident in the current results (Nimalaratne *et al.*, 2011; Sharp, Becker, & Hettich, 2004).

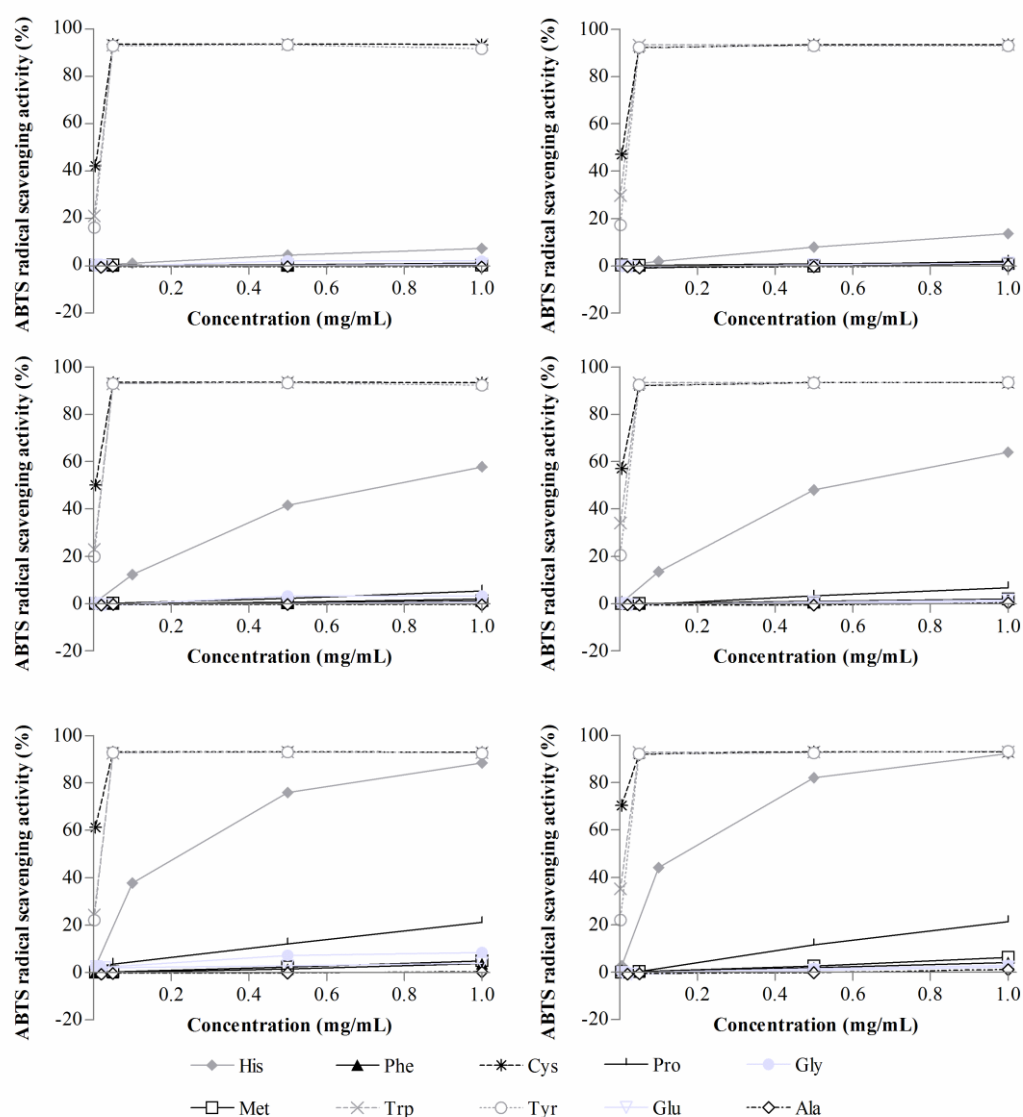


Figure 4.24 ABTS radical scavenging activity after 1min (top), 8min (middle) and 31min (bottom) incubation. Left : amino acid was dissolved in 0.1M ammonium acetate (pH=8). Right: amino acids were dissolved in dH₂O.

Solutions of free cysteine were reported by Marcuse (1962) to behave pro-oxidatively in a linoleic acid system and he also refers that similar results were found in earlier studies (Marcuse, 1961). However, he also states that it is often possible to invert pro-oxidant activities or enhance weak antioxidant activities of amino acids by changing experimental conditions, and thus states that any antioxidative effect is only a “potential” one. By changing the conditions cysteine was found also to be an antioxidant (Marcuse, 1962). Since then cysteine has been found to inhibit oxidation of methyl linoleate in a model system (Karel, Tannenbaum, Wallace, & Maloney, 1966), free cysteine from whey has been demonstrated to act antioxidatively (Tong, Sasaki, McClements, & Decker, 2000), and a cysteine solution showed radical scavenging activities in an

ORAC-FL assay (Hernández-Ledesma *et al.*, 2005). The potential for cysteine to act as a radical scavenger lies in sulfhydryl-group (Meucci & Mele, 1997). Furthermore, Meucci & Mele (1997) found cysteine to display antioxidant abilities at very low concentration in an ABTS-based assay, and that cysteine demonstrated an inhibitory pattern quite similar to that of non-protein antioxidant like ascorbic acid and trolox. In the current study cysteine showed similar behaviour to trolox, yet trolox demonstrated slightly higher activity at 0.005mg/mL (data not shown). This could be due to solvent differences.

Histidine also demonstrated ABTS radical scavenging activity, however, the kinetic behaviour was somewhat different as it reacted much slower but clearly increased during the incubation (Figure 4.24). Within the tested timeframe (31min) the most concentrated solution approached the same activity (92% in water and 88% in ammonium acetate –almost corresponding to assays max capacity) as cysteine, tryptophan and tyrosine. Histidine is well known for its multifaceted antioxidant activities and like cysteine, tryptophan and tyrosine the origin of histidine's antioxidative properties lies in its sidechain structure as the imidazole group enables histidine to scavenge radicals by proton/hydrogen donation (Chan, Decker, Lee, & Butterfield, 1994). Meucci & Mele (1997) did not see an effect of monomeric histidine on the ABTS radical, yet several other studies have found free histidine to inhibit lipid oxidation and scavenge free radicals (Hernández-Ledesma *et al.*, 2005; Karel *et al.*, 1966; Marcuse, 1962). This small inconsistency might be related to the fact that histidine can act both as a hydrogen donor as well as an acceptor at physiological pH but also has a tendency to invert to a pro-oxidant at high concentrations (Udenigwe & Aluko, 2011). Also for histidine the water-based solution exhibited higher ABTS radical scavenging activity compared to the ammonium acetate solution at the same concentration and time of incubation (significantly different for all time point at concentration ≥ 0.1 mg/mL, $P < 0.05$).

The antioxidant activity of the individual amino acids is affected by a number of factors. One important factor is the pH of the reaction environment as it may affect the optimum for the antioxidant activity of the amino acids. This is not considered to be a problem as the assays applied to the sample were carried out in exactly the same way as the assays applied on the amino acid solutions needed for the comparisons. However, antioxidant capacity was found to decrease with decreasing pH in a linoleic acid model system (Marcuse, 1962). If this is also the case in the aqueous and ammonium acetate environments, it would be expected that the assays carried out in ammonium acetate (pH=8) would show higher antioxidant activity than the same solution in assays carried out in dH₂O. As this was not the case for any of the amino acids mentioned above other factors may also be affecting the systems. In conclusion attention must be paid to solvent and buffer differences when comparing to previous findings.

As for many antioxidants the concentration of the amino acids are essential for their degree of antioxidant activity and at very high concentrations it might even turn into pro-oxidative activities (Marcuse, 1962). From the current results it is believed that the highly active tryptophan, tyrosine, cysteine and histidine

contributed additively to the radical scavenging activities detected in the crude and SEC fractionated aqueous ≤ 10 kDa NCCPP extracts and that the concentration of these as free amino acids is below the concentration for conversion to pro-oxidative reactivity.

Among the remaining tested amino acid solutions only proline (water and ammonium acetate), glycine (ammonium acetate) and methionine (water) in 1mg/mL demonstrated ABTS radical scavenging activity above 5% after 31min incubation. Proline and methionine have previously been found to scavenge free radicals (Damgaard *et al.*, 2015; Hernández-Ledesma *et al.*, 2005). However, assuming a similar content of the individual amino acids in aqueous ≤ 10 kDa extracts of any NCCPP these amino acids are unlikely to contribute to the overall radical scavenging antioxidant properties of such a product – at least on a short term basis.

Of the last three tested amino acids showing $< 5\%$ ABTS radical scavenging activity, phenylalanine was selected for testing, due to previous reports of strong antioxidant properties (Carrasco-Castilla *et al.*, 2012; Di Bernardini *et al.*, 2011) among others playing a positive role in the scavenging of H_2O_2 likely attributed to by the aromatic ring (Udenigwe & Aluko, 2011) and for displaying *in vitro* radical scavenging activity (ORAC-FL) in free form (Hernández-Ledesma *et al.*, 2005). The unexpected absent result could be assay-related, as Meucci & Mele (1997) also did not find free phenylalanine capable of inhibiting the ABTS radical. Glutamic acid was the most dominant in the amino acid profile of the commercial NCCPPs (Paper I – Figure 3), and it is also frequently mentioned in connection with antioxidant peptides but mainly in regards to metal chelation (Saiga *et al.*, 2003; Zhu *et al.*, 2013). Glutamic acid may, however, donate its acidic hydrogen near neutral pH and thereby potentially be able to scavenge free radicals.

Like for ABTS radical scavenging solutions of cysteine, tryptophan and tyrosine were also the ones standing out in the reducing power assay (Figure 4.25). Cysteine demonstrated markedly higher reducing power capacities than the other two amino acids in both water and ammonium acetate, however, OD_{700} was only significantly different between the two solvents for tryptophan. The strong reducing power of cysteine is again associated with the sulfhydryl-group (Udenigwe & Aluko, 2011). The minute reducing power activity displayed by tyrosine is most likely to be caused by the turbidity of the solution, mentioned above. Based on these results it is considered unlikely that any of the tested amino acids other than cysteine would contribute noticeably to the overall *in vitro* reducing power, and that the contribution from free cysteine might be limited due to low content in the aqueous ≤ 10 kDa NCCPP extracts.

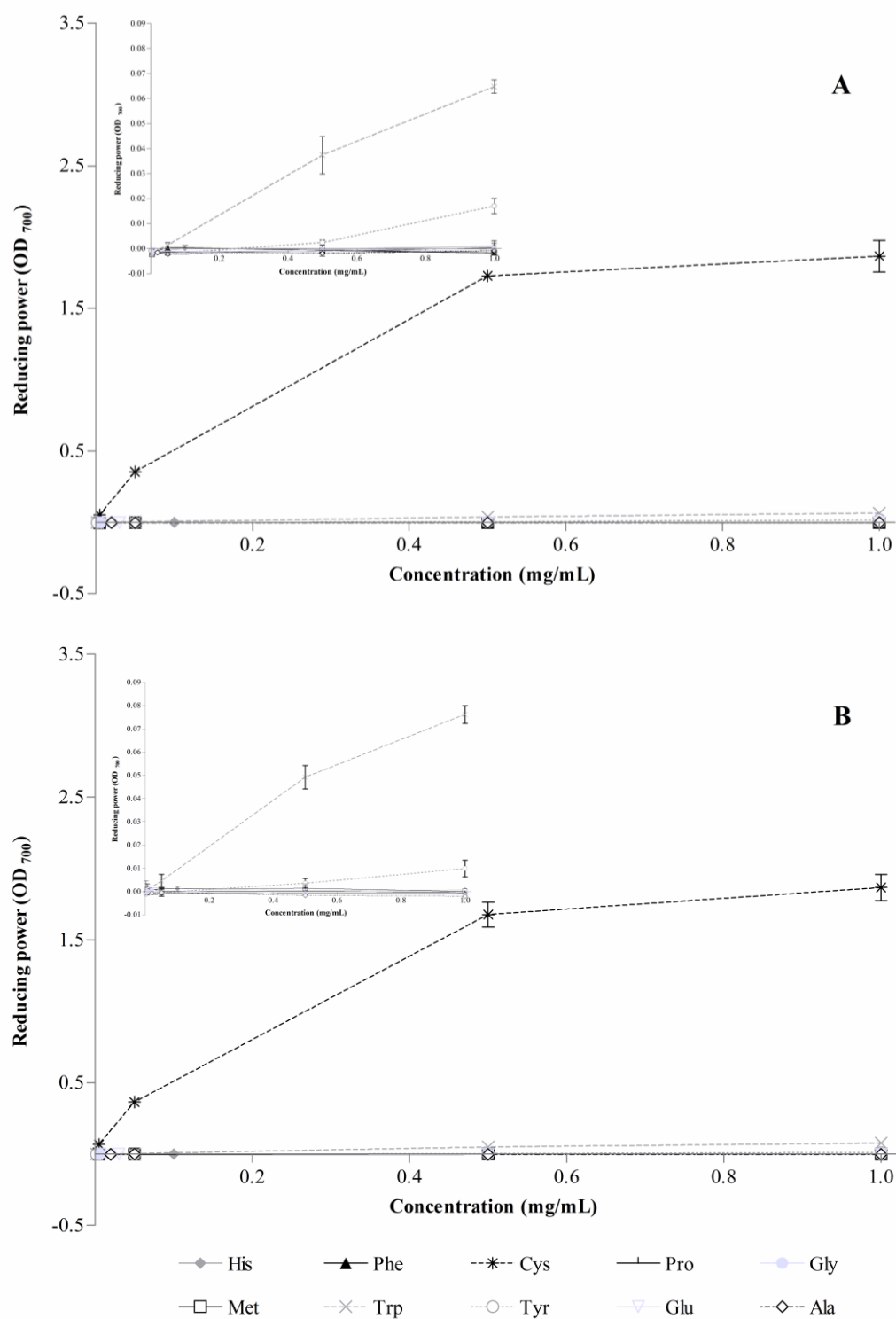


Figure 4.25 Reducing power activity of selected amino acids dissolved in A: ammonium acetate pH=8 and B: dH₂O. For each plot a zoom of the y-axis is provided in the insert.

In conclusion, it is believed that the tested free amino acids demonstrating ABTS radical scavenging and reducing power activity contribute to the activities measured in the aqueous ≤ 10 kDa NCCPP extracts and in the SEC separated extract fractions although to a lesser extent due to dilution. However, the signals detected in the current experiments do not add up to the full antioxidant capacities measured in the aqueous ≤ 10 kDa extracts. Thus, other compounds such as modified amino acids e.g. S-nitrosocysteine (Kanner, 1979), peptides e.g. carnosine (Gil-Agustí *et al.*, 2008), nitrite and ascorbate must also be contributing to varying extent.

Chapter 5

Summarising discussion and conclusion

As the results of the current PhD work has been thoroughly discussed during their presentation in the preceding parts of Chapter 4 and in the Paper I-III, the following aims to summarise the main findings by addressing the interface between the studies mainly focusing on the three working hypothesis presented in the introduction in Chapter 1.

Hypothesis 1 - During the production of cooked pork product with nitrite, antimicrobial and antioxidant compounds are formed

Overall the current PhD originally set out to examine antimicrobial and antioxidative activities in NCCPPs, and in order to do so *in vitro* measurements of these activities was carried out. Independently of differences in the type and processing condition, including type and amount of additives, of the tested NCCPPs no growth inhibitory activities were detected. Although, the lack of detected antimicrobial activities stands in contrast to the literature emphasising a higher microbiological stability in nitrite-cured meat products, (Myers *et al.*, 2016; Osterbauer *et al.*, 2017; Sindelar & Milkowski, 2012) the vast majority of the published evidence seems to be based on inoculation studies or model studies of nitrite or similar antimicrobial agents added to the growth medium rather than antimicrobial activity tests on isolated meat fractions. Thus, it may be the case that antimicrobial activities in NCCPPs are related to the curing process but the active compounds might somehow be associated with the meat matrix and thus could not be measured in the aqueous extracts. Therefore, it must be stressed that although the two tested aqueous NCCPP fractions (supernatant of meat/water homogenate and aqueous $\leq 10\text{kDa}$ fraction) demonstrated no antimicrobial activities, a follow-up inoculation study is required in order to fully establish whether the result is in fact due to a complete lack of antimicrobial activities in all the tested NCCPPs. Alternatively, as pointed out in Chapter 4.3.1, the tested NCCPP fractions could contain antimicrobial compounds but they were merely tested in too low concentrations to generate a response. In contrast to antimicrobial activities, *in vitro* antioxidant activity was detected in the aqueous $\leq 10\text{kDa}$ extracts of all the different types of NCCPPs under investigation. On a theoretical basis it is already known that the addition of nitrite and ascorbate in the production of cooked pork products will lead to the presence of antioxidative species. Regarding NCCPPs both heating in general and heating at higher temperature will lead to a decrease in residual inorganic nitrite (Honikel, 2008), which could mean that more of the antioxidatively active NO is formed. The importance of ascorbate was very clear from the current investigations, however, whether antioxidant activity correlated with added or residual ascorbate varied between the sample categories, sample treatments (SEC) and storage.

Furthermore, the interplay between added ascorbate and added nitrite seem to greatly affect the detected *in vitro* activity. This could be due to mutual reactions leading to formation of known as well as unknown reactions products of increased or decreased antioxidative properties, compared to the individual reactants. When focusing on shelf-life extending additives it would appear that the circumstances under which antioxidative activities take place in NCCPPs play a vital role for the type of mechanisms and potentially also type of compounds exerting the antioxidative activities.

Decades ago it was reported that nitrite reacts readily with peptides (Cassens *et al.*, 1979). Consequently, it was initially strongly hypothesised that compounds with strong shelf-life extending properties formed during production of cooked pork products with nitrite could very well be reaction products of protein origin. Yet, the conducted experiments e.g. LC-MS and biotin switch could not aid in confirming the importance of nitrite-induced modified peptides for the detected antioxidant activities. However, as the biotin switch did not succeed in producing credible results (signal in negative control) and the LC-MS methods were not optimised to search selectively for nitrosations and nitrations, S-nitrosocysteine containing peptides formed in reactions between nitrite and meat proteins and previously reported to be antioxidative (Kanner, 1979) may have been formed and contributed to the *in vitro* antioxidant properties of the aqueous ≤ 10 kDa extracts of the tested NCCPPs. In addition to potentially containing active modified peptides, residual additives and active species hereof the extracts were generally found to constitute very complex mixture of peptides/proteins. Through the characterisation of the extracts it became clear that the *in vitro* antioxidant activities had to originate from a highly diverse selection of compounds, and that small peptides and certain amino acids e.g. tyrosine, tryptophan, histidine, proline and cysteine may have been of great importance for the *in vitro* antioxidative properties measured in the tested NCCPPs. So in conclusion it can be said that antioxidant activity in ≤ 10 kDa NCCPP extracts was due to strong synergetic effects of protein and non-protein, anti- and pro-oxidant compounds to a wide extent originating from processing.

Hypothesis 2 - There are optimal processing conditions for the formation of these antimicrobial and antioxidant compounds

With active compounds generated during processing, attention should be paid to determining the optimal condition for their formation. As lipid oxidation can be perceived as a self-perpetuating process, initiation of oxidative reactions by any mechanism (e.g. metals or free radicals) may generate compounds that can initiate new oxidative reactions or contribute to the elongation of ongoing reactions. Thus, all types of antioxidative actions are of vital importance for the oxidative balance of the NCCPP and consequently the evaluation of the optimal processing conditions for generation of an optimal antioxidative response should be made across all three applied *in vitro* antioxidative assays. Although, antioxidant activity based on chelating of iron (and other metals) is of general importance, such activity was not found to be related to curing. Thus, the iron

chelating assay was not included in the estimation of optimal processing conditions concerning addition of the two curing agents. As added nitrite/ascorbate to the model products correlated positive with normalised ABTS radical scavenging activity and initial reducing power activity, but not with reducing power of the crude aqueous ≤ 10 kDa extracts, addition of ≤ 150 ppm nitrite and ≤ 600 ppm ascorbate constituted the test-level, at which the best overall antioxidative response was obtained. In addition to indicating an optimal curing agent addition level the results also show that in order to obtain a high degree of antioxidative reactions without converting to pro-oxidative activities, the nitrite to ascorbate ratio should be considered when producing NCCPPs. Solely adding ascorbate (on the expense of conventional nitrite-related effects on colour and taste) could perhaps be a solution to achieve high antioxidative activities, as the reducing power dilution curve for the aqueous ≤ 10 kDa extracts from the sausage only added high levels of ascorbic acid (s0/2000) did not deflect in the same manor as the samples added high levels of ascorbate as well as nitrite. Ascorbate/ascorbic acid is considered a non-hazardous (EFSA, 2015a; FDA, 2018) additive and could thus be added relatively unrestrained to NCCPPs (European Union, 2011). However, the literature states the antioxidative properties may turn pro-oxidative under certain conditions (Haak, Raes, & De Smet, 2009; Villaverde, Parra, *et al.*, 2014). Furthermore, the antimicrobial properties accomplished with the addition of nitrite – although not demonstrated in the current study – may likely be severely altered with this strategy. For example ascorbic acid has been found to possess antimicrobial properties against a variety of bacteria (Cross *et al.*, 2003; Giannuzzi & Zaritzky, 1996; Juven & Kanner, 1986; Tabak, Armon, Rosenblat, Stermer, & Neeman, 2003; Vilchèze, Hartman, Weinrick, & Jacobs, 2013). However, to the best of my knowledge no newer research on anti-botulinal activities in meat as a result of ascorbate/ascorbic acid addition without nitrite are available. Less recent studies reported of some reduction in spore germination in the presence of ascorbic acid when tested in medium (Eller, Edwards, & Wynne, 1968), while no considerably different effect on botulinal toxin formation was evident on ascorbate addition compared to combined ascorbate/nitrite addition or nitrite alone to wiener sausages (Bowen, Cervený, & Deibel, 1974). Thus, the suggested strategy may be inferior to nitrite treatments in regard to inhibiting *C. botulinum*.

Once new or optimised methods for the detection of potential antimicrobial activities has been established, as suggested in Chapter 4.3.1, it would be necessary to do a re-evaluation to set new levels required for the best compromise between antioxidative and antimicrobial activities. Lastly, it must be kept in mind that matrix effects may affect the oxidative (and microbiological) activities in the entire NCCPPs potentially changing the suggestion for optimal processing conditions, in regard to curing agents, presented above.

Other aspects of processing than addition of curing agents were also found to affect the active antioxidant aqueous ≤ 10 kDa NCCPP extracts. When comparing the model hams and sausages a clear effect of processing in the intensity of 215nm SEC peaks that aligned with the active antioxidant fraction-peaks was evident. Since processing appeared to influence the protein portion of the SEC profile, it may be speculated that different antioxidative peptides may be formed as a result of different processing methods. As the

current results show that certain types of interventions associated with processing affect the measured *in vitro* antioxidant activity in the aqueous $\leq 10\text{kDa}$ NCCPP extract, the next appropriate step would be to also investigate the effect of other processing parameters such as cooking time and temperature.

Achieving a certain centre temperature as well as holding time at this temperature, is key to obtaining a microbial safe product, but this will naturally also have other consequences on e.g. residual nitrite and ascorbate. The supply of energy occurring during heat treatment may also act as an activation-energy for the formation of so far unknown reaction products (e.g. between nitrite and meat proteins) with antioxidative and antimicrobial properties. As different reactions, may require different activation energies in order to proceed, the mixture of reaction products in a multi substrate system such as meat will vary with temperature. Also the longer the heat is applied, the longer the heat/energy dependent reactions may proceed. Additionally heating leads to an increase in lipid oxidation (Boles, 2010). Ergo, the process of determining optimal processing conditions is not merely a matter of finding the optimal balance of antioxidative and antimicrobial compounds but also to keep the level of “oxidation initiator”, e.g. free radicals, at a level where the newly formed or already existing antioxidative compounds can keep them at bay (Baardseth, Bjerke, Aaby, & Mielnik, 2005).

Hypothesis 3 - There are optimal storage conditions to retain the activity of antimicrobial and antioxidant compounds in nitrite cured pork products

It was the ambition to investigate and establish the optimal storage conditions for retaining the activity of identified antimicrobial and antioxidant compounds. The effects of storage temperature, as well as access to oxidation initiators as light and oxygen were to be tested. So far, it was established that cold storage over time resulted in an increase in normalised ABTS and partly in initial reducing power in the aqueous $\leq 10\text{kDa}$ NCCPP extracts. Furthermore, the results showed a storage time (and additive concentration) dependent formation of unidentified compounds contributing to reducing power and iron chelating activity. However, during this time of cold storage oxidative species in the entire pork meat products will also have increased hampering the potential of any new antioxidants requiring time to form. Combined this may still be a part of the reason for the prolonged shelf-life of nitrite cured products, yet it does not provide evidence for any recommendations for optimal storage conditions for retaining antimicrobial and antioxidant activity in NCCPPs. Furthermore, regular refrigerated storage (short time for the $\leq 10\text{kDa}$ extracts of the lower level of nitrite/ascorbate addition and longer for the hams, h500/2000₂₇₆) resulted in a decrease in a SEC peak expected of potentially representing ascorbic acid. As ascorbic acid was found to be of great importance for the *in vitro* antioxidant activities optimised storage conditions for the entire hams will have to be determined.

Chapter 6

Future work

The current PhD has established that antioxidant compounds are formed during the production of NCCPPs but the nature of the compounds is multiple and occasionally inconclusive. Furthermore, the formation of these compounds was affected by processing and storage. However, in order (for the industry) to fully draw benefits and possibly apply the new knowledge obtained in this PhD work a complete characterisation of the ≤ 10 kD extracts are required. By establishing the composition of the extracts, the compounds and mechanisms behind the detected *in vitro* antioxidant activity can be investigated and ultimately contribute to the optimisation of the nitrite-curing process.

With the many and rapid advancements in the field of MS it would be logic to turn to further exploration of MS-based methods for extract characterisation and quantification. The next step would then be to conduct a comprehensive analysis including testing of purified constituents individually and in different combinations, in order to gain in-depth understanding of chemical interactions constituting the foundation of the detected *in vitro* antioxidant activity. Should the aqueous ≤ 10 kDa extract constituent characterisation lead to the disclosure of until now unknown reaction product(s) of nitrite and meat constituents, possessing strong antioxidant or even antimicrobial properties, several perspectives require further elucidation. First of all the reaction kinetics and especially the concentration dependencies of the compound(s) needs to be investigated. As frequently observed, antioxidative compounds may convert their reactivity from antioxidative to pro-oxidative at higher concentrations. This information is essential if efforts are to be made for optimising the production of NCCPPs for enhanced formation of such active compound(s) with the lowest possible addition of nitrite. Simultaneous investigations of toxicity should be conducted, so that a search for preservatives less harmful than nitrite are suspected of being, reaches its purpose. This is especially important considering that other reaction products of nitrite and meat constituent namely some N-nitrosamines have been found to be carcinogenic (Parthasarathy & Bryan, 2012).

A full characterisation would likely have to include alternative methods for further clean up and fractionation of the extracts to be explored. As it was evident from the presented results that residual ascorbic acid are greatly but not exclusively contributing to the detected *in vitro* antioxidant activity, methods for its removal from the aqueous ≤ 10 kDa extracts requires further attention. This would enable verification of the extent of the antioxidant contribution from other extract constituents. If the ascorbic acid-free fraction were then found to be equally important for *in vitro* antioxidant activity in aqueous ≤ 10 kDa NCCPP extracts, a full characterisation of extract composition would be even more prudent. New ways to pursue purification of constituent compounds based on nitrite-induced modifications may also be considered. As part of this, further optimisation of the SDS-PAGE (Appendix A) and re-attempting biotin switch immunoblotting could

be contemplated, as some samples unsuited for dot blotting may behave differently after electrophoretic separation.

Other fractions than the aqueous ≤ 10 kDa could certainly also have an impact on the prolonged oxidative and microbial shelf-life of NCCPPs, and extending the investigations to include these would therefore also be highly relevant. Ultimately, the antioxidative and antimicrobial findings would have to be tested and optimised in genuine and different types of NCCPPs as indicated in Chapter 3.2.2.

On several occasions there appeared to be a correlation between ascorbic acid and certain types of *in vitro* antioxidant activities, but one specific discovery especially contributed with new knowledge for the understanding of antioxidative actions in NCCPPs. When measuring residual ascorbic acid in samples of varying ascorbate addition during storage in connection with Paper III, the measured content behaved different than expected (See Figure 4.4 in Chapter 4.2.1). The findings were supported by the behaviour of the reducing power measurements as well as several measurement repetitions on aqueous ≤ 10 kDa extracts and the actual meat, however, a clear explanation could not be extrapolated. Thus, it is of great importance to further investigate the cause of the observed drop to provided better understanding of the antioxidative actions taking place in NCCPPs. Furthermore, it is worth considering testing other methods for the determination of residual ascorbic acid in order to compare to the HPLC results. Especially, as the BCA protein determination method in the preliminary investigations (Figure 4.22 – Chapter 4.5.1) seemed to detect (residual) ascorbic acid to increase with ascorbate addition at day 0 as it was initially expected. However, it must be emphasised that the BCA method in its current form was not thoroughly tested for its application as an ascorbic acid detection method for NCCPP aqueous extracts.

Although, addition of nitrite to meat products has been extensively investigated in the current PhD and through the past several decades the (partial) list above underlines that much is still to be explored. With the many valid arguments for reducing nitrite addition it is clear that this topic still deserves a lot of attention.

References

- Adler-Nissen, J., Ekgreen, M. H., & Risum, J. (2014). Practical use of nitrite and basis for dosage in the manufacture of meat products. Natinoal Food Institute, Technical University of Denmark.
- Andersen, H. J., Saaby Johansen, H., Shek, C. K., & Skibsted, L. H. (1990). Nitric oxide exchange in nitrosylmyoglobin. *Zeitschrift Für Lebensmittel-Untersuchung Und -Forschung*, 191, 293–298.
- AOAC Official Methods of Analysis. (1996). Crude protein in meat. Block digestion method 39.1.19 981.10.
- Ashworth, J., Hargreaves, L. L., & Jarvis, B. (1973). The production of an antimicrobial effect in pork heated with sodium nitrite under simulated commercial pasteurization conditions. *Journal of Food Technology*, 8(4), 477–484.
- Barbieri, G., Bergamaschi, M., Barbieri, G., & Franceschini, M. (2013). Kinetics of nitrite evaluated in a meat product. *Meat Science*, 93(2), 282–286.
- Baron, C. P., Berner, L., Skibsted, L. H., & Refsgaard, H. H. F. (2005). Evaluation of activity of selected antioxidants on proteins in solution and in emulsions. *Free Radical Research*, 39(7), 777–785.
- Baron, C. P., & Andersen, H. J. (2002). Myoglobin-induced lipid oxidation. A review. *Journal of Agricultural and Food Chemistry*, 50(14), 3887–3897.
- Bartberger, M. D., Mannion, J. D., Powell, S. C., Stamler, J. S., Houk, K. N., & Toone, E. J. (2001). S–N dissociation energies of S-nitrosothiols: On the origins of nitrosothiol decomposition rates. *Journal of the American Chemical Society*, 123(36), 8868–8869.
- Bendich, A., Machlin, L. J., Scandurra, O., Burton, G. W., & Wayner, D. D. M. (1986). The antioxidant role of vitamin C. *Advances in Free Radical Biology & Medicine*, 2(2), 419–444.
- Berardo, A., De Maere, H., Stavropoulou, D. A., Rysman, T., Leroy, F., & De Smet, S. (2016). Effect of sodium ascorbate and sodium nitrite on protein and lipid oxidation in dry fermented sausages. *Meat Science*, 121, 359–364.
- Biltoft-Jensen, A. P., Kørup, K., Christensen, T., Eriksen, K., Ygil, K. H., & Fagt, S. (2016). Køds rolle i kosten.
- Binker, E. F., & Kolari, O. E. (1975). The history and use of nitrate and nitrite in the curing of meat. *Food and Cosmetics Toxicology*, 13(6), 655–661.
- Blanchard-Fillion, B., Prou, D., Polydoro, M., Spielberg, D., Tsika, E., Wang, Z., ... Ischiropoulos, H.

- (2006). Metabolism of 3-nitrotyrosine induces apoptotic death in dopaminergic cells. *Journal of Neuroscience*, 26(23), 6124–6130.
- Bligh, E. G., & Dyer, W. J. (1959). A rapid method for total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology*, 37, 911–917.
- Boles, J. A. (2010). Thermal Processing. In F. Toldrá (Ed.), *Handbook of Meat Processing* (1st ed., pp. 169–183). Ames, IA, USA: Blackwell Publishing.
- Boman, H. G. (2003). Antibacterial peptides: basic facts and emerging concepts. *Journal of Internal Medicine*, 254(3), 197–215.
- Bowen, V. G., Cervený, J. G., & Deibel, R. H. (1974). Effect of sodium ascorbate and sodium nitrite on toxin formation of *Clostridium botulinum* in wieners. *Applied Microbiology*, 27(3), 605–606.
- Brady, J. W. (2013). *Introductory food chemistry* (1st ed.). Ithaca and London: Comstock Publishing Associates.
- Brannan, R. G., Connolly, B. J., & Decker, E. A. (2001). Peroxynitrite: a potential initiator of lipid oxidation in food. *Trends in Food Science & Technology*, 12(5–6), 164–173.
- Brown, T. B., & Stevens, M. F. G. (1975). Confirmation of the site of nitrosation in tryptophan derivatives by α -Chymotrypsin. *Journal of the Chemical Society Perkin Transactions I*, (22), 2357–2360.
- Buchanan, R. L., & Solberg, M. (1972). Interaction of sodium nitrate, oxygen and pH on growth of *Staphylococcus aureus*. *Journal of Food Science*, 37(1), 81–85.
- Budryn, G., Gałazka-Czarnecka, I., Brzozowska, E., Grzelczyk, J., Mostowski, R., Żyżelewicz, D., ... Pérez-Sánchez, H. (2018). Evaluation of estrogenic activity of red clover (*Trifolium pratense* L.) sprouts cultivated under different conditions by content of isoflavones, calorimetric study and molecular modelling. *Food Chemistry*, 245, 324–336.
- Burney, S., Caulfield, J. L., Niles, J. C., Wishnok, J. S., & Tannenbaum, S. R. (1999). The chemistry of DNA damage from nitric oxide and peroxynitrite. *Mutation Research*, 424(1–2), 37–49.
- Baardseth, P., Bjerke, F., Aaby, K., & Mielnik, M. (2005). A screening experiment to identify factors causing rancidity during meat loaf production. *European Food Research and Technology*, 221(5), 653–661. 7
- Cammack, R., Joannou, C. L., Cui, X.-Y., Martinez, C. T., Maraj, S. R., & Hughes, M. N. (1999). Nitrite and nitrosyl compounds in food preservation. *Biochimica Et Biophysica Acta*, 1411(2–3), 475–488.

- Carlsen, C. U., Møller, J. K. S., & Skibsted, L. H. (2005). Heme-iron in lipid oxidation. *Coordination Chemistry Reviews*, 249(3–4), 485–498.
- Carrasco-Castilla, J., Hernández-Álvarez, A. J., Jiménez-Martínez, C., Jacinto-Hernández, C., Alaiz, M., Girón-Calle, J., ... Dávila-Ortiz, G. (2012). Antioxidant and metal chelating activities of peptide fractions from phaseolin and bean protein hydrolysates. *Food Chemistry*, 135(3), 1789–1795.
- Cassens, R. G., Greaser, M. L., Ito, T., & Lee, M. (1979). Reactions of nitrite in meat. *Food Technology*, 33(7), 46–57.
- Castellano, P., Mora, L., Escudero, E., Vignolo, G., Aznar, R., & Toldrá, F. (2016). Antilisterial peptides from Spanish dry-cured hams: Purification and identification. *Food Microbiology*, 59, 133–141.
- Chan, W. K. M., Decker, E. A., Lee, J. B., & Butterfield, D. A. (1994). EPR spin-trapping studies of the hydroxyl radical scavenging activity of carnosine and related dipeptides. *Journal of Agricultural and Food Chemistry*, 42(7), 1407–1410.
- Chen, H.-M., Muramoto, K., Yamauchi, F., & Nokihara, K. (1996). Antioxidant activity of designed peptides based on the antioxidative peptide isolated from digests of a soybean protein. *Journal of Agricultural and Food Chemistry*, 44(9), 2619–2623.
- Chen, L.-N., Shi, Q., Zhang, X.-M., Zhang, B.-Y., Lv, Y., Chen, C., ... Dong, X.-P. (2015). Optimization of the isolation and enrichment of S-nitrosylated proteins from brain tissues of rodents and humans with various prion diseases for iTRAQ-based proteomics. *International Journal of Molecular Medicine*, 35(1), 125–134.
- Chiueh, C. C., & Rauhala, P. (1999). The redox pathway of S -nitrosoglutathione, glutathione and nitric oxide in cell to neuron communications. *Free Radical Research*, 31(6), 641–650.
- Cross, J. B., Currier, R. P., Torrace, D. J., Vanderberg, L. A., Wagner, G. L., & Gladen, P. D. (2003). Killing of *Bacillus* spores by aqueous dissolved oxygen, ascorbic acid, and copper ions. *Applied and Environmental Microbiology*, 69(4), 2245–2252.
- Crow, J. P., & Beckman, J. S. (1995). Quantitation of protein tyrosine, 3-nitrotyrosine, and 3-aminotyrosine utilizing HPLC and iIntrinsic ultraviolet absorbance. *Methods*, 7(1), 116–120.
- Damgaard, T., Lametsch, R., & Otte, J. (2015). Antioxidant capacity of hydrolyzed animal by-products and relation to amino acid composition and peptide size distribution. *Journal of Food Science and Technology*, 52(10), 6511–6519.

- de Castro, R. J. S., & Sato, H. H. (2015). Biologically active peptides: Processes for their generation, purification and identification and applications as natural additives in the food and pharmaceutical industries. *Food Research International*, 74, 185–198.
- Dean, R. T., Hunt, J. V., Grant, A. J., Yamamoto, Y., & Niki, E. (1991). Free radical damage to proteins: The influence of the relative localization of radical generation, antioxidants, and target proteins. *Free Radical Biology & Medicine*, 11(2), 161–168.
- Decker, E. A., Warner, K., Richards, M. P., & Shahidi, F. (2005). Measuring antioxidant effectiveness in food. *Journal of Agricultural and Food Chemistry*, 53(10), 4303–4310.
- Delatour, T., Fenaille, F., Parisod, V., Richoz, J., Vuichoud, J., Mottier, P., & Buetler, T. (2007). A comparative study of proteolysis methods for the measurement of 3-nitrotyrosine residues: Enzymatic digestion versus hydrochloric acid-mediated hydrolysis. *Journal of Chromatography B*, 851(1–2), 268–276.
- Di Bernardini, R., Rai, D. K., Bolton, D., Kerry, J., O'Neill, E., Mullen, A. M., ... Hayes, M. (2011). Isolation, purification and characterization of antioxidant peptidic fractions from a bovine liver sarcoplasmic protein thermolysin hydrolyzate. *Peptides*, 32(2), 388–400.
- Di Luccia, A., la Gatta, B., Nicastro, A., Petrella, G., Lamacchia, C., & Picariello, G. (2015). Protein modifications in cooked pork products investigated by a proteomic approach. *Food Chemistry*, 172, 447–455.
- Duan, X., Ocen, D., Wu, F., Li, M., Yang, N., Xu, J., ... Xu, X. (2014). Purification and characterization of a natural antioxidant peptide from fertilized eggs. *Food Research International*, 56, 18–24.
- Duncan, M. W. (2003). A review of approaches to the analysis of 3-nitrotyrosine. *Amino Acids*, 25(3–4), 351–361.
- Dunn, B. P., & Stich, H. F. (1984). Determination of free and protein-bound N-nitrosoproline in nitrite-cured meat products. *Food and Chemical Toxicology*, 22(8), 609–613.
- EFSA. (2003). Opinion of the scientific panel on biological hazards on the request from the commission related to the effects of nitrites/nitrates on the microbiological safety of meat products. *EFSA Journal*, 14, 1–31.
- EFSA. (2015a). Scientific Opinion on the re-evaluation of ascorbic acid (E 300), sodium ascorbate (E 301) and calcium ascorbate (E 302) as food additives. *EFSA Journal*, 13(5), 4087.

- EFSA. (2015b). The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2014. *EFSA Journal*, 13(12), 4329.
- EFSA. (2017). Scientific Opinion: Re-evaluation of potassium nitrite (E 249) and sodium nitrite (E 250) as food additives. *EFSA Journal*, 15(6), 4786.
- Elias, R. J., Kellerby, S. S., & Decker, E. A. (2008). Antioxidant Activity of Proteins and Peptides. *Critical Reviews in Food Science and Nutrition*, 48(5), 430–441.
- Eller, C., Edwards, F. F., & Wynne, E. S. (1968). Sporicidal action of auto-oxidized ascorbic acid for *Clostridium*. *Applied Microbiology*, 16(2), 349–354.
- Emi-Miwa, M., Okitani, A., & Fujimaki, M. (1976). Comparison of the fate of nitrite added to whole meat, meat fractions and model Systems. *Agricultural and Biological Chemistry*, 40(7), 1387–1392.
- Escudero, E., Mora, L., Fraser, P. D., Aristoy, M.-C., & Toldrá, F. (2013). Identification of novel antioxidant peptides generated in Spanish dry-cured ham. *Food Chemistry*, 138(2–3), 1282–1288.
- European Union. (2006). Directive 2006/52/EC of the European Parliament and of the Council of 5 July 2006 amending Directive 95/2/EC on food additives other than colours and sweeteners and Directive 94/35/EC on sweeteners for use in foodstuffs. *Official Journal of the European Union*, L204/10.
- European Union. (2008). Regulation (EC) No 1333/2008 of the European Parliament and of the Council of 16 December 2008 on food additives. *Official Journal of the European Union*, L354/16.
- European Union. (2011). Commission Regulation (EU) No 1129/2011 of 11 November 2011 amending Annex II to Regulation (EC) No 1333/2008 of the European Parliament and of the Council by establishing a Union list of food additives. *Official Journal of the European Union*, L295/1.
- European Union. (2015). Commission Decision (EU) 2015/826 of 22 May 2015 concerning national provisions notified by Denmark on the addition of nitrite to certain meat products. *Official Journal of the European Union*, L130/10.
- Farvin, K. H. S., Andersen, L. L., Nielsen, H. H., Jacobsen, C., Jakobsen, G., Johansson, I., & Jessen, F. (2014). Antioxidant activity of Cod (*Gadus morhua*) protein hydrolysates: In vitro assays and evaluation in 5% fish oil-in-water emulsion. *Food Chemistry*, 149, 326–334.
- FDA. (2018). CFR - Code of Federal Regulations Title 21 - Food and drugs Chapter I - Food and drug administration department of health and human services subchapter B -Food for human consumption (continued) Part 182: substances generally recognised as safe. Retrieved March 22, 2018, from

https://www.ecfr.gov/cgi-bin/text-idx?SID=6082cad3c61bf6145800cbb68691fd65&mc=true&tpl=/ecfrbrowse/Title21/21cfr182_main_02.tpl

- Feng, X., Li, C., Jia, X., Guo, Y., Lei, N., Hackman, R. M., ... Zhou, G. (2016). Influence of sodium nitrite on protein oxidation and nitrosation of sausages subjected to processing and storage. *Meat Science*, 116, 260–267.
- Feng, X., Li, C., Ullah, N., Hackman, R. M., Chen, L., & Zhou, G. (2015). Potential biomarker of myofibrillar protein oxidation in raw and cooked ham: 3-Nitrotyrosine formed by nitrosation. *Journal of Agricultural and Food Chemistry*, 63(51), 10957–10964.
- Fiddler, W., Pensabene, J. W., Gates, R. A., & Adam, R. (1998). Nitrosamine formation in processed hams as related to reformulated elastic rubber netting. *Journal of Food Science*, 63(2), 276–278.
- Forrester, M. T., Foster, M. W., Benhar, M., & Stamler, J. S. (2009). Detection of protein S-nitrosylation with the biotin-switch technique. *Free Radical Biology and Medicine*, 46(2), 119–126.
- Fox, J. B. (1966). The chemistry of meat pigments. *Journal of Agricultural and Food Chemistry*, 14(3), 207–210.
- Fox, J. B. (1974). Role of cure accelerators. In *Proceedings of the Meat Industry Research Conference* (pp. 17–21). Arlington, VA, USA: American Meat Institute Foundation.
- Freybler, L. A., Gray, J. I., Asghar, A., Booren, A. M., Pearson, A. M., & Buckley, D. J. (1993). Nitrite stabilization of lipids in cured pork. *Meat Science*, 33(1), 85–96.
- Fødevarestyrelsen. (2017). Listeria. Retrieved July 25, 2017, from <https://www.foedevarestyrelsen.dk/Leksikon/Sider/Listeria.aspx>
- Giannuzzi, L., & Zaritzky, N. E. (1996). Effect of ascorbic acid in comparison to citric and lactic acid on *Listeria monocytogenes* inhibition at refrigeration temperatures. *LWT - Food Science and Technology*, 29(3), 278–285.
- Gibson, A. M., Roberts, T. A., & Robinson, A. (1984). Factors controlling the growth of *Clostridium botulinum* types A and B in pasteurized cured meats VI. Nitrite monitoring during storage of pasteurized pork slurries. *Journal of Food Technology*, 19(1), 29–44.
- Gil-Agustí, M., Esteve-Romero, J., & Carda-Broch, S. (2008). Anserine and carnosine determination in meat samples by pure micellar liquid chromatography. *Journal of Chromatography A*, 1189(1–2), 444–450.

- Gilbert, J., Knowles, M. E., & McWeeny, D. J. (1975). Formation of C- and S- nitroso compounds and their further reactions. *Journal of the Science of Food and Agriculture*, 26, 1785–1791.
- Goutefongea, R., Cassens, R. G., & Woolford, G. (1977). Distribution of sodium nitrite in adipose tissue during curing. *Journal of Food Science*, 42(6), 1637–1641.
- Heinz, G., & Hautzinger, P. (2007). Meat processing technology for small- to medium-scale producers. Food and Agriculture Organization of the United Nations (FAO). Retrieved from <http://www.fao.org/3/a-ai407e.pdf>
- Helwich, B., Christensen, J., & Müller, L. (2016). Annual report on zoonoses in Denmark 2016. National Food Institute, Technical University of Denmark.
- Hernández-Ledesma, B., Dávalos, A., Bartolomé, B., & Amigo, L. (2005). Preparation of antioxidant enzymatic hydrolysates from α -lactalbumin and β -lactoglobulin. Identification of active peptides by HPLC-MS/MS. *Journal of Agricultural and Food Chemistry*, 53(3), 588–593.
- Herrmann, S. S., Granby, K., & Duedahl-Olesen, L. (2015). Formation and mitigation of N-nitrosamines in nitrite preserved cooked sausages. *Food Chemistry*, 174, 516–526.
- Hloucalová, P., Skládanka, J., Horký, P., Klejdus, B., Pelikán, J., & Knotová, D. (2016). Determination of phytoestrogen content in fresh-cut legume forage. *Animals*, 6(12), 43.
- Honikel, K.-O. (2008). The use and control of nitrate and nitrite for the processing of meat products. *Meat Science*, 78(1–2), 68–76.
- Hospital, X. F., Hierro, E., & Fernández, M. (2014). Effect of reducing nitrate and nitrite added to dry fermented sausages on the survival of Salmonella Typhimurium. *Food Research International*, 62, 410–415.
- Hustad, G. O., Cervený, J. G., Trenk, H., Deibel, R. H., Kautter, D. A., Fazio, T., ... Kolari, O. E. (1973). Effect of sodium nitrite and sodium nitrate on botulinal toxin production and nitrosamine formation in wieners. *Applied Microbiology*, 26(1), 22–26.
- Haak, L., Raes, K., & De Smet, S. (2009). Effect of plant phenolics, tocopherol and ascorbic acid on oxidative stability of pork patties. *Journal of the Science of Food and Agriculture*, 89(8), 1360–1365.
- Igene, J. O., Yamauchi, K., Pearson, A. M., Gray, J. I., & Aust, S. D. (1985). Mechanisms by which nitrite inhibits the development of warmed-over flavour (WOF) in cured meat. *Food Chemistry*, 18(1), 1–18.
- Incze, K., Farkas, J., Mihályi, V., & Zukál, E. (1974). Antibacterial effect of cysteine-nitrosothiol and

- possible precursors thereof. *Applied Microbiology*, 27(1), 202–205.
- Ito, T., Cassens, R. G., Greaser, M. L., Lee, M., & Izumi, K. (1983). Lability and reactivity of nonheme protein-bound nitrite. *Journal of Food Science*, 48(4), 1204–1207.
- Iwase, H. (2000). Use of an amino acid in the mobile phase for the determination of ascorbic acid in food by high-performance liquid chromatography with electrochemical detection. *Journal of Chromatography A*, 881(1–2), 317–326.
- Izumi, K. (1992). Reaction of nitrite with ascorbic acid or ascorbic acid-2-derivatives. *Journal of Food Science*, 57(5), 1066–1067.
- Izumi, K., Cassens, R. G., & Greaser, M. L. (1989). Reaction of nitrite with ascorbic acid and its significant role in nitrite-cured food. *Meat Science*, 26(2), 141–153.
- Jacobsen, C., Adler-Nissen, J., & Meyer, A. S. (1999). Effect of ascorbic acid on iron release from the emulsifier interface and on the oxidative flavor deterioration in fish oil enriched mayonnaise. *Journal of Agricultural and Food Chemistry*, 47(12), 4917–4926.
- Jaffrey, S. R., Erdjument-Bromage, H., Ferris, C. D., Tempst, P., & Snyder, S. H. (2001). Protein S-nitrosylation: A physiological signal for neuronal nitric oxide. *Nature Cell Biology*, 3(2), 193–197.
- Jampel, H. D. (1994). Determination of protein concentration in aqueous humor. *Journal of Glaucoma*, 3(1), 5–11.
- Joosen, A. M. C. P., Kuhnle, G. G. C., Aspinall, S. M., Barrow, T. M., Lecommandeur, E., Azqueta, A., ... Bingham, S. A. (2009). Effect of processed and red meat on endogenous nitrosation and DNA damage. *Carcinogenesis*, 30(8), 1402–1407.
- Juven, B. J., & Kanner, J. (1986). Effect of ascorbic, isoascorbic and dehydroascorbic acids on the growth and survival of *Campylobacter-jejuni*. *Journal of Applied Bacteriology*, 61(4), 339–345.
- Kall, M. A., & Andersen, C. (1999). Improved method for simultaneous determination of ascorbic acid and dehydroascorbic acid, isoascorbic acid and dehydroisoascorbic acid in food and biological samples. *Journal of Chromatography B*, 730(1), 101–111.
- Kalyanaraman, B., & Singh, R. J. (1998). S-nitrosoglutathione inhibits SOD/H₂O₂-dependent lipid peroxidation. *Faseb Journal*, 12(5), A1008.
- Kanner, J. (1979). S-nitrosocysteine (RSNO), an effective antioxidant in cured meat. *Journal of the American Oil Chemists' Society*, 56(2), 74–76.

- Kanner, J. (1994). Oxidative processes in meat and meat products: Quality implications. *Meat Science*, 36(1–2), 169–189.
- Kanner, J., Ben-Gera, I., & Berman, S. (1980). Nitric-oxide myoglobin as an inhibitor of lipid oxidation. *Lipids*, 15(11), 944–948.
- Kanner, J., & Juven, B. J. (1980). S-nitrosocysteine as an antioxidant, color-developing, and anticlostridial agent in comminuted turkey meat. *Journal of Food Science*, 45(5), 1105–1112.
- Karel, M., Tannenbaum, S. R., Wallace, D. H., & Maloney, H. (1966). Autoxidation of methyl linoleate in freeze-dried model systems. III. Effects of added amino acids. *Journal of Food Science*, 31(6), 892–896.
- King, A. M., Glass, K. A., Milkowski, A. L., Seman, D. L., & Sindelar, J. J. (2016). Modeling the impact of ingoing sodium nitrite, sodium ascorbate, and residual nitrite concentrations on growth parameters of *Listeria monocytogenes* in cooked, cured pork sausage. *Journal of Food Protection*, 79(2), 184–193.
- Klimczak, I., & Gliszczynska-Świgło, A. (2015). Comparison of UPLC and HPLC methods for determination of vitamin C. *Food Chemistry*, 175, 100–105.
- Kubberød, G., Cassens, R. G., & Greaser, M. L. (1974). Reaction of nitrite with sulfhydryl groups of myosin. *Journal of Food Science*, 39(6), 1228–1230.
- Kutnink, M. A., & Omaye, S. T. (1987). Determination of ascorbic acid, erythorbic acid, and uric acid in cured meats by high performance liquid chromatography. *Journal of Food Science*, 52(1), 53–56.
- Laemmli, U. K. (1970). Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature*, 227(5259), 680–685.
- Landbrug & Fødevarer. (2017). Statistik 2016 - Svinekød. Landbrug og Fødevarer.
- Larsson, S. C., Bergkvist, L., & Wolk, A. (2006). Processed meat consumption, dietary nitrosamines and stomach cancer risk in a cohort of Swedish women. *International Journal of Cancer*, 119(4), 915–919.
- Larsson, S. C., & Wolk, A. (2012). Red and processed meat consumption and risk of pancreatic cancer: meta-analysis of prospective studies. *British Journal of Cancer*, 106(3), 603–607.
- Lee, M., Cassens, R. G., & Fennema, O. R. (1981). Effect of metal ions on residual nitrite. *Journal of Food Processing and Preservation*, 5(4), 191–205.
- Lowry, O. H., Rosebrough, N. J., Farr, L., & Randall, R. J. (1951). Protein measurement with the Folin

- phenol reagent. *Journal of Biological Chemistry*, 193(1), 265–275.
- Lund, M. N., & Baron, C. P. (2010). Protein oxidation in foods and food quality. In L. H. Skibsted, J. Risbo, & M. L. Andersen (Eds.), *Chemical deterioration and physical instability of food and beverages* (1st ed., pp. 33–69). Cambridge, United Kingdom: Woodhead Publishing Limited.
- Lund, M. N., Heinonen, M., Baron, C. P., & Estévez, M. (2011). Protein oxidation in muscle foods: A review. *Molecular Nutrition & Food Research*, 55, 83–95.
- Mac Donald, B., Gray, J. I., Kakuda, Y., & Lee, M. L. (1980). Role of nitrite in cured meat flavor: Chemical analysis. *Journal of Food Science*, 45(4), 889–892.
- Mangialasche, F., Polidori, M. C., Monastero, R., Ercolani, S., Camarda, C., Cecchetti, R., & Mecocci, P. (2009). Biomarkers of oxidative and nitrosative damage in Alzheimer's disease and mild cognitive impairment. *Ageing Research Reviews*, 8(4), 285–305.
- Marcuse, R. (1961). Zur Wirkung von Aminosäuren bei der Fett-Oxydation II: Untersuchungen mit Linoleat. *Fette, Seifen, Anstrichmittel*, 63(10), 940–944.
- Marcuse, R. (1962). The effect of some amino acids on the oxidation of linoleic acid and its methyl ester. *Journal of the American Oil Chemists Society*, 39(2), 97–103.
- Market Data Forecast. (2016). Europe processed meat market by processing technology (fresh-processed meat, raw-cooked meat, pre-cooked meat, raw-fermented sausages, cured meat, dried meat), by meat type (poultry, beef, pork and mutton), and by region - Industry analysis, size, share,. Retrieved March 14, 2018, from <https://www.marketdataforecast.com/market-reports/europe-processed-meat-market-867/>
- Marshall, T., & Williams, K. M. (1992). Drug interference in the 2,2'-bicinchoninic acid protein assay. *Biochemical Society Transactions*, 20(1), 40S–40S.
- Martinaud, A., Mercier, Y., Marinova, P., Tassy, C., Gatellier, P., & Renerre, M. (1997). Comparison of oxidative processes on myofibrillar proteins from beef during maturation and by different model oxidation systems. *Journal of Agricultural and Food Chemistry*, 45(7), 2481–2487.
- Merino, L., Örnemark, U., & Toldrá, F. (2017). Analysis of nitrite and nitrate in foods: Overview of chemical, regulatory and analytical aspects. *Advances in Food and Nutrition Research*, 81, 65–107.
- Meucci, E., & Mele, M. C. (1997). Amino acids and plasma antioxidant capacity. *Amino Acids*, 12(3–4), 373–377.

- Miersch, S., & Mutus, B. (2005). Protein S-nitrosation: Biochemistry and characterization of protein thiol–NO interactions as cellular signals. *Clinical Biochemistry*, 38(9), 777–791.
- Milkowski, A., Garg, H. K., Coughlin, J. R., & Bryan, N. S. (2010). Nutritional epidemiology in the context of nitric oxide biology: A risk–benefit evaluation for dietary nitrite and nitrate. *Nitric Oxide*, 22(2), 110–119.
- Miranda, K. M., Nims, R. W., Thomas, D. D., Espey, M. G., Citrin, D., Bartberger, M. D., ... Wink, D. A. (2003). Comparison of the reactivity of nitric oxide and nitroxyl with heme proteins - A chemical discussion of the differential biological effects of these redox related products of NOS. *Journal of Inorganic Biochemistry*, 93(1–2), 52–60.
- Mora, L., Escudero, E., Fraser, P. D., Aristoy, M.-C., & Toldrá, F. (2014). Proteomic identification of antioxidant peptides from 400 to 2500Da generated in Spanish dry-cured ham contained in a size-exclusion chromatography fraction. *Food Research International*, 56, 68–76.
- Mordor Intelligence. (2017). Europe Processed Meat Market - Growth, Trends and Forecasts (2018 - 2023). Retrieved March 14, 2018, from <https://www.mordorintelligence.com/industry-reports/europe-processed-meat-market>
- Morrissey, P. A., & Tichivangana, J. Z. (1985). The antioxidant activities of nitrite and nitrosylmyoglobin in cooked meats. *Meat Science*, 14(3), 175–190.
- Myers, M. I., Sebranek, J. G., Dickson, J. S., Shaw, A. M., Tarté, R., Adams, K. R., & Neibuhr, S. (2016). Implications of decreased nitrite concentrations on *Clostridium perfringens* outgrowth during cooling of ready-to-eat meats. *Journal of Food Protection*, 79(1), 153–156.
- Mäkinen, M., Kähkönen, M., & Hopia, A. (2001). Ascorbic acid and ascorbyl palmitate have only minor effect on the formation and decomposition of methyl linoleate hydroperoxides. *European Journal of Lipid Science and Technology*, 103(10), 683–687.
- Møller, J. K. S., & Skibsted, L. H. (2002). Nitric oxide and myoglobins. *Chemical Reviews*, 102(4), 1167–1178.
- Møller, J. K. S., Sosniecki, L., & Skibsted, L. H. (2002). Effect of nitrosylmyoglobin and saturated fatty acid anions on metmyoglobin-catalyzed oxidation of aqueous methyl linoleate emulsions. *Biochimica et Biophysica Acta*, 1570(2), 129–134.
- Najafian, L., & Babji, A. S. (2012). A review of fish-derived antioxidant and antimicrobial peptides: Their production, assessment, and applications. *Peptides*, 33(1), 178–185.

- Nakai, H., Cassens, R. G., Greaser, M. L., & Woolford, G. (1978). Significance of the reaction of nitrite with tryptophan. *Journal of Food Science*, 43(6), 1857–1860.
- Niki, E. (1991). Action of ascorbic acid as a radical scavenger of active and stable oxygen radicals. *American Journal of Clinical Nutrition*, 54(6), 1119S–1124S.
- Nimalaratne, C., Lopes-Lutz, D., Schieber, A., & Wu, J. (2011). Free aromatic amino acids in egg yolk show antioxidant properties. *Food Chemistry*, 129(1), 155–161.
- Nordin, H. R. (1969). The depletion of added sodium nitrite in ham. *Canadian Institute of Food Technology Journal*, 2(2), 79–85.
- Nováková, L., Solich, P., & Solichová, D. (2008). HPLC methods for simultaneous determination of ascorbic and dehydroascorbic acids. *TrAC Trends in Analytical Chemistry*, 27(10), 942–958.
- Olsman, W. J., & van Leeuwen, C. M. (1977). The assay of protein-bound nitrite in meat products. *Zeitschrift Für Lebensmittel-Untersuchung Und -Forschung*, 164(4), 239–242.
- Osterbauer, K. J., King, A. M., Seman, D. L., Milkowski, A. L., Glass, K. A., & Sindelar, J. J. (2017). Effects of nitrite and erythorbate on *Clostridium perfringens* growth during extended cooling of cured ham. *Journal of Food Protection*, 80(10), 1697–1704.
- Ozyurt, V. H., & Otles, S. (2017). Determination of 3-nitrotyrosine in food protein suspensions. *Talanta*, 171, 81–89.
- Parthasarathy, D. K., & Bryan, N. S. (2012). Sodium nitrite: The “cure” for nitric oxide insufficiency. *Meat Science*, 92(3), 274–279.
- Pearson, A. M., & Gillett, T. A. (1996). *Processed meats* (3rd ed.). New York, NY, USA: Chapman & Hall.
- Pegg, R. B., & Shahidi, F. (1997). Unraveling the chemical identity of meat pigments. *Critical Reviews in Food Science and Nutrition*, 37(6), 561–589.
- Pegg, R. B., & Shahidi, F. (2004). *Nitrite curing of meat: The N-nitrosamine problem and nitrite alternatives* (1st ed.). Trumbull, CT, USA: Wiley-Blackwell.
- Pensabene, J. W., Feinberg, J. I., Piotrowski, E. G., & Fiddler, W. (1979). Occurrence and determination of N-nitrosoproline and N-nitrosopyrrolidine in cured meat products. *Journal of Food Science*, 44(6), 1700–1702.
- Pérez-Rodríguez, M. L., Bosch-Bosch, N., & Garcíá-Mata, M. (1996). Monitoring nitrite and nitrate residues

- in frankfurters during processing and storage. *Meat Science*, 44(1–2), 65–73.
- Pichner, R., Hechelmann, H., Steinrueck, H., & Gareis, M. (2006). Shigatoxin producing *Escherichia coli* (STEC) in conventionally and organically produced salami products. *Fleischwirtschaft*, 86(10), 112–114.
- Pourfarzam, M., Movahedian, A., Sarrafzadegan, N., Basati, G., & Samsamshariat, S. Z. (2013). Association between plasma myeloperoxidase and free 3-nitrotyrosine levels in patients with coronary artery disease. *International Journal of Clinical Medicine*, 4(3), 158–164.
- Przybyciel, M., & Majors, R. E. (2002). Phase collapse in reversed-phase liquid chromatography. *LC-GC North America*, 20(6), 516–523.
- Reddy, D., Lancaster, J. R., & Cornforth, D. P. (1983). Nitrite inhibition of *Clostridium botulinum*: Electron spin resonance detection of iron-nitric oxide complexes. *Science*, 221(4612), 769–770.
- Ren, J., Zhao, M., Shi, J., Wang, J., Jiang, Y., Cui, C., ... Xue, S. J. (2008). Purification and identification of antioxidant peptides from grass carp muscle hydrolysates by consecutive chromatography and electrospray ionization-mass spectrometry. *Food Chemistry*, 108(2), 727–736.
- Robinson, A., Gibson, A. M., & Roberts, T. A. (1982). Factors controlling the growth of *Clostridium botulinum* types A and B in pasteurized, cured meats V. Prediction of toxin production. *Journal of Food Technology*, 17(6), 727–744.
- Sacchetti, G., Mattia, C. D., Pittia, P., & Martino, G. (2008). Application of a radical scavenging activity test to measure the total antioxidant activity of poultry meat. *Meat Science*, 80, 1081–1085.
- Saiga, A., Tanabe, S., & Nishimura, T. (2003). Antioxidant activity of peptides obtained from porcine myofibrillar proteins by protease treatment. *Journal of Agricultural and Food Chemistry*, 51(12), 3661–3667.
- Saito, K., Jin, D.-H., Ogawa, T., Muramoto, K., Hatakeyama, E., Yasuhara, T., & Nokihara, K. (2003). Antioxidative properties of tripeptide libraries prepared by the combinatorial chemistry. *Journal of Agricultural and Food Chemistry*, 51(12), 3668–3674.
- Santarelli, R., Pierre, F., & Corpet, D. (2008). Processed meat and colorectal cancer: A review of epidemiologic and experimental evidence. *Nutrition and Cancer*, 60(2), 131–144.
- Sarmadi, B. H., & Ismail, A. (2010). Antioxidative peptides from food proteins: A review. *Peptides*, 31(10), 1949–1956.

- Sebranek, J. G. (2009). Basic curing ingredients. In R. Tarte (Ed.), *Ingredients in Meat Products* (3rd ed., pp. 1–23). New York, NY: Springer New York.
- Sebranek, J. G., & Bacus, J. N. (2007). Cured meat products without direct addition of nitrate or nitrite: what are the issues? *Meat Science*, 77(1), 136–147.
- Sebranek, J. G., & Fox, J. B. (1985). A review of nitrite and chloride chemistry: Interactions and implications for cured meats. *Journal of the Science of Food and Agriculture*, 36(11), 1169–1182.
- Sebranek, J. G., Jackson-Davis, A. L., Myers, K. L., & Lavieri, N. A. (2012). Beyond celery and starter culture: Advances in natural/organic curing processes in the United States. *Meat Science*, 92(3), 267–273.
- Sharp, J. S., Becker, J. M., & Hettich, R. L. (2004). Analysis of protein solvent accessible surfaces by photochemical oxidation and mass spectrometry. *Analytical Chemistry*, 76(3), 672–683.
- Shichi, H., Page, T., Sahouri, M. J., & Shin, D. H. (1997). Microplate assay of ascorbic acid in aqueous humor with bicinchoninic acid. *Journal of Ocular Pharmacology and Therapeutics*, 13(3), 201–206.
- Shigenaga, M. K., Lee, H. H., Blount, B. C., Christen, S., Shigeno, E. T., Yip, H., & Ames, B. N. (1997). Inflammation and NO_x-induced nitration: Assay for 3-nitrotyrosine by HPLC with electrochemical detection. *Proceedings of the National Academy of Sciences*, 94(7), 3211–3216.
- Shihabi, Z. K., & Dyers, R. D. (1988). Protein analysis with bicinchoninic acid. *Annals of Clinical and Laboratory Science*, 18(3), 235–239.
- Sindelar, J. J., & Milkowski, A. L. (2011). Sodium nitrite in processed meat and poultry meats: A review of curing and examining the risk benefit of its use. *AMSA White Paper Series*, 3, 1–14.
- Sindelar, J. J., & Milkowski, A. L. (2012). Human safety controversies surrounding nitrate and nitrite in the diet. *Nitric Oxide*, 26(4), 259–266.
- Skibsted, L. H. (2011). Nitric oxide and quality and safety of muscle based foods. *Nitric Oxide*, 24(4), 176–183.
- Slocum, T. L., & Deupree, J. D. (1991). Interference of biogenic amines with the measurement of proteins using bicinchoninic acid. *Analytical Biochemistry*, 195(1), 14–17.
- Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., ... Klenk, D. C. (1985). Measurement of protein using bicinchoninic acid. *Analytical Biochemistry*, 150(1), 76–85.

- Soladoye, O. P., Juárez, M. L., Aalhus, J. L., Shand, P., & Estévez, M. (2015). Protein oxidation in processed meat: Mechanisms and potential implications on human health. *Comprehensive Reviews in Food Science and Food Safety*, 14(2), 106–122.
- Sucu, N., Ünlü, A., Tamer, L., Aytacoglu, B., Ercan, B., Dikmengil, M., & Atik, U. (2003). 3-nitrotyrosine in atherosclerotic blood vessels. *Clinical Chemistry and Laboratory Medicine*, 41(1), 23–25.
- Sullivan, G. A., & Sebranek, J. G. (2012). Nitrosylation of myoglobin and nitrosation of cysteine by nitrite in a model system simulating meat curing. *Journal of Agricultural and Food Chemistry*, 60(7), 1748–1754.
- Sun, W., Zhao, H., Zhao, Q., Zhao, M., Yang, B., Wu, N., & Qian, Y. (2009). Structural characteristics of peptides extracted from Cantonese sausage during drying and their antioxidant activities. *Innovative Food Science & Emerging Technologies*, 10(4), 558–563.
- Tabak, M., Armon, R., Rosenblat, G., Stermer, E., & Neeman, I. (2003). Diverse effects of ascorbic acid and palmitoyl ascorbate on *Helicobacter pylori* survival and growth. *FEMS Microbiology Letters*, 224(2), 247–253.
- Teixeira, D., Fernandes, R., Prudêncio, C., & Vieira, M. (2016). 3-Nitrotyrosine quantification methods: Current concepts and future challenges. *Biochimie*, 125, 1–11.
- Thermo Fisher Scientific. (n.d.). Chemistry of Protein Assays. Retrieved June 1, 2017, from <https://www.thermofisher.com/dk/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/chemistry-protein-assays.html>
- Thermo Fisher Scientific. (n.d.). Instructions - Modified Lowry Protein Assay Kit (23240). Retrieved March 3, 2018, from <https://www.funakoshi.co.jp/data/datasheet/PCC/23240.pdf>
- Thermo Fisher Scientific. (n.d.). Pierce BCA Protein Assay Kit - Thermo Fisher Scientific. Retrieved January 27, 2018, from <https://www.thermofisher.com/order/catalog/product/23225>
- Thompson, J. W., Forrester, M. T., Moseley, M. A., & Foster, M. W. (2013). Solid-phase capture for the detection and relative quantification of S-nitrosoproteins by mass spectrometry. *Methods*, 62(2), 130–137.
- Tong, L. M., Sasaki, S., McClements, D. J., & Decker, E. A. (2000). Antioxidant activity of whey in a salmon oil emulsion. *Journal of Food Science*, 65(8), 1325–1329.
- Tricker, A. R., & Kubacki, S. J. (1992). Review of the occurrence and formation of non-volatile N-nitroso

- compounds in foods. *Food Additives and Contaminants*, 9(1), 39–69. Retrieved from
- Udenigwe, C. C., & Howard, A. (2013). Meat proteome as source of functional biopeptides. *Food Research International*, 54(1), 1021–1032.
- Udenigwe, C. C., & Aluko, R. E. (2011). Chemometric analysis of the amino acid requirements of antioxidant food protein hydrolysates. *International Journal of Molecular Sciences*, 12(12), 3148–3161.
- Valero, A., Carrasco, E., Pérez-Rodríguez, F., García-Gimeno, R. M., & Zurera, G. (2006). Growth/no growth model of *Listeria monocytogenes* as a function of temperature, pH, citric acid and ascorbic acid. *European Food Research and Technology*, 224(1), 91–100.
- Valls, F., Sancho, M. T., Fernández-Muiño, M. A., Alonso-Torre, S., & Checa, M. A. (2002). High-pressure liquid chromatographic determination of ascorbic acid in cooked sausages. *Journal of Food Protection*, 65(11), 1771–1774.
- Velasco, J., Dobarganes, C., & Márquez-Ruiz, G. (2010). Oxidative rancidity in foods and food quality. In L. H. Skibsted, J. Risbo, & M. L. Andersen (Eds.), *Chemical deterioration and physical instability of food and beverages* (1st ed., pp. 3–32). Cambridge, United Kingdom: Woodhead Publishing Limited.
- Vilchèze, C., Hartman, T., Weinrick, B., & Jacobs, W. R. (2013). *Mycobacterium tuberculosis* is extraordinarily sensitive to killing by a vitamin C-induced Fenton reaction. *Nature Communications*, 4, 1881.
- Villaverde, A., Morcuende, D., & Estévez, M. (2014). Effect of curing agents on the oxidative and nitrosative damage to meat proteins during processing of fermented sausages. *Journal of Food Science*, 79(7), C1331–C1342.
- Villaverde, A., Parra, V., & Estévez, M. (2014). Oxidative and nitrosative stress induced in myofibrillar proteins by a hydroxyl-radical-generating system: Impact of nitrite and ascorbate. *Journal of Agricultural and Food Chemistry*, 62(10), 2158–2164.
- Vossen, E., & De Smet, S. (2015). Protein oxidation and protein nitration influenced by sodium nitrite in two different meat model systems. *Journal of Agricultural and Food Chemistry*, 63(9), 2550–2556.
- Wanasundara, P. K. J. P. D., & Shahidi, F. (2005). Antioxidants: Science, technology, and applications. In F. Shahidi (Ed.), *Bailey's Industrial Oil and Fat Products* (6th ed., pp. 431–489). Hoboken, NJ, USA: John Wiley & Sons, Inc.

- Wettasinghe, M., & Shahidi, F. (1997). Antioxidant activity of preformed cooked cured-meat pigment in a β -carotene/linoleate model system. *Food Chemistry*, 58(3), 203–207.
- Wiechelman, K. J., Braun, R. D., & Fitzpatrick, J. D. (1988). Investigation of the bicinchoninic acid protein assay: Identification of the groups responsible for color formation. *Analytical Biochemistry*, 175(1), 231–237.
- Willemot, C., Fillion-Delorme, N., & Wood, D. F. (1987). Effect of nitrite on lipid degradation during storage of cooked pork meat. *Canadian Institute of Food Science and Technology Journal*, 20(2), 70–74.
- Williams, K. M., Arthur, S. J., Burrell, G., Kelly, F., Phillips, D. W., & Marshall, T. (2003). An evaluation of protein assays for quantitative determination of drugs. *Journal of Biochemical and Biophysical Methods*, 57(1), 45–55.
- Woolford, G., Cassens, R. G., Greaser, M. L., & Sebranek, J. G. (1976). The fate of nitrite: Reaction with protein. *Journal of Food Science*, 41(3), 585–588.
- Xing, L.-J., Hu, Y.-Y., Hu, H.-Y., Ge, Q.-F., Zhou, G.-H., & Zhang, W.-G. (2016). Purification and identification of antioxidative peptides from dry-cured Xuanwei ham. *Food Chemistry*, 194, 951–958.
- Yang, H., Zhang, Y., & Pöschl, U. (2010). Quantification of nitrotyrosine in nitrated proteins. *Analytical and Bioanalytical Chemistry*, 397(2), 879–886.
- Zacharis, C. K., & Tzanavaras, P. D. (2012). Preservatives. In L. M. L. Nollet & F. Toldra (Eds.), *Food Analysis by HPLC* (3rd ed., pp. 529–550). Boca Raton, FL, USA: CRC Press, Taylor and Francis Group.
- Zhang, G., Ross, C. R., & Blecha, F. (2000). Porcine antimicrobial peptides: New prospects for ancient molecules of host defense. *Veterinary Research*, 31(3), 277–296. Retrieved from
- Zhang, Y., Fonslow, B. R., Shan, B., Baek, M.-C., & Yates, J. R. (2013). Protein analysis by shotgun/bottom-up proteomics. *Chemical Reviews*, 113(4), 2343–2394.
- Zhu, C.-Z., Zhang, W.-G., Zhou, G.-H., Xu, X.-L., Kang, Z.-L., & Yin, Y. (2013). Isolation and identification of antioxidant peptides from Jinhua ham. *Journal of Agricultural and Food Chemistry*, 61(6), 1265–1271.
- Zion Market Research. (2017). Processed meat market (cured processed, uncured processed and others) by meat type (poultry, pork, mutton, beef and other); by types (chilled processed meat, frozen processed

meat, canned/preserved meat and meat products and frozen processed red meat): Gl. Retrieved March 14, 2018, from <https://www.zionmarketresearch.com/market-analysis/processed-meat-market>

Appendix

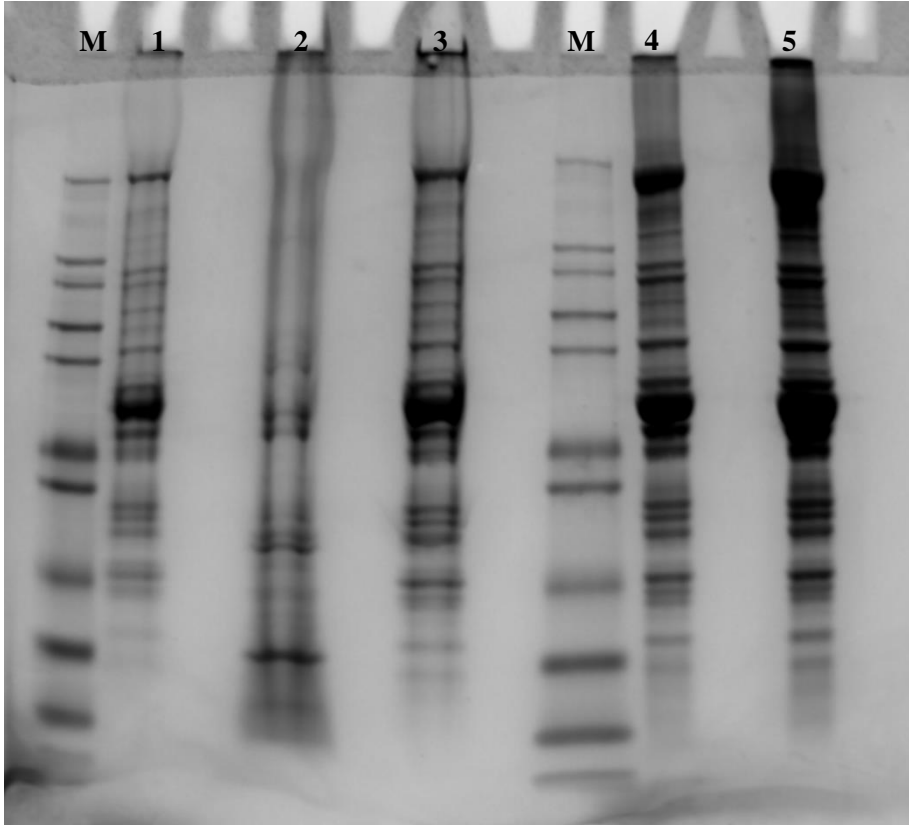
Appendix A: SDS-PAGE

Appendix B: Shotgun analysis of selected SEC fractions of SH₀

Appendix C: Selected ChemSpider hits for the m/z 431 ion

Appendix A

– SDS-PAGE



Gel: RunBlue 12% (Expedeon, San Diego, CA, USA)

Staining: InstantBlue (Expedeon)

Marker: Mark-12™ (life Technologies, Thermo Fisher Scientific)

Sample: Meat:water homogenate of SH₀

Lane code	Sample description	Loaded amount
M	Marker	10μL
1	Total meat:water homogenate (TCA precipitated)	10μL
2	Water-soluble fraction of meat:water homogenate (TCA precipitated)	20μL
3	Fraction of meat:water homogenate not soluble in water(TCA precipitated)	10μL
4	Total meat:water homogenate	10μL
5	Fraction of meat:water homogenate not soluble in water	10μL

Appendix B

– Shotgun analysis of selected SEC fractions of SH₀

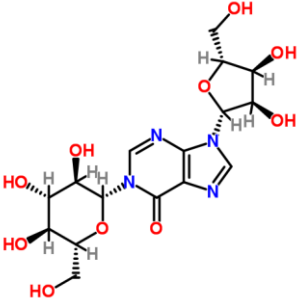
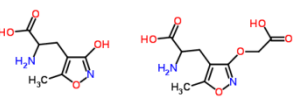
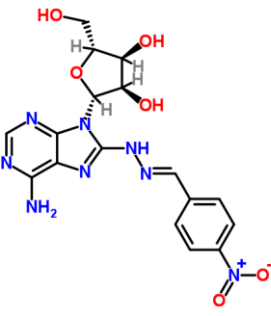
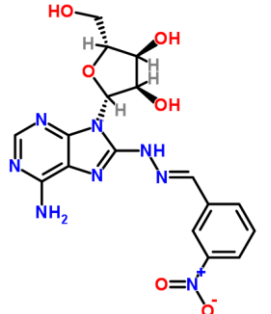
Peptide description	Peak I				Peak II	Peak III	Peak IV
	C9	C10	C11	C12	D10	D5	E9
Serotransferrin OS=Sus scrofa GN=TF PE=1 SV=2	X	X	X	X	X	X	X
Trypsin OS=Sus scrofa PE=1 SV=1	X	X	X	X	X	X	X
Alpha-1-antichymotrypsin 2 OS=Sus scrofa GN=SERPINA3-2 PE=2 SV=1	X	X	X	X	X	X	X
Serum albumin OS=Sus scrofa GN=ALB PE=4 SV=1	X	X	X	X	X	X	X
Alpha-1-antitrypsin OS=Sus scrofa GN=SERPINA1 PE=3 SV=1	X	X	X		X	X	X
Complement C3 OS=Sus scrofa GN=C3 PE=1 SV=2	X	X	X	X	X	X	X
Inter-alpha-trypsin inhibitor heavy chain H4 OS=Sus scrofa GN=ITI4 PE=4 SV=2	X	X	X	X	X	X	X
contaminant_CTRB_BOVIN	X	X		X		X	X
Actin, cytoplasmic 1 OS=Sus scrofa GN=ACTB PE=2 SV=1	X	X		X	X	X	
Junction plakoglobin OS=Sus scrofa GN=Jup PE=2 SV=1		X	X	X			
Cardiac muscle alpha actin 1 OS=Sus scrofa GN=ACTC1 PE=2 SV=1		X		X			
Enolase OS=Sus scrofa GN=ENO1 PE=3 SV=1		X		X			
Desmoglein-1 OS=Sus scrofa GN=DSG1 PE=3 SV=1		X		X			
contaminant_ALBU_BOVIN		X	X		X	X	X
Annexin OS=Sus scrofa GN=ANXA2 PE=3 SV=2		X		X		X	
Serine-protein kinase ATM (Fragment) OS=Sus scrofa GN=ATM PE=4 SV=1		X					
Peroxiredoxin-2 (Fragment) OS=Sus scrofa GN=PRDX2 PE=2 SV=1		X	X				
Pyruvate kinase (Fragment) OS=Sus scrofa GN=PKM PE=2 SV=2		X		X			
Glyceraldehyde-3-phosphate dehydrogenase OS=Sus scrofa GN=GAPDH PE=1 SV=4		X					
Fibrinogen alpha chain (Fragment) OS=Sus scrofa GN=FGA PE=1 SV=1		X					X
Transthyretin OS=Sus scrofa GN=TTR PE=1 SV=1			X	X	X		
Retinol-binding protein 4 OS=Sus scrofa GN=RBP4 PE=2 SV=1			X				
Tropomyosin alpha-4 chain OS=Sus scrofa GN=TPM4 PE=2 SV=3			X				
Tropomyosin alpha-3 chain OS=Sus scrofa GN=TPM3 PE=2 SV=2			X	X			
Tropomyosin alpha-1 chain OS=Sus scrofa GN=TPM1 PE=3 SV=1			X				
Troponin C, skeletal muscle OS=Sus scrofa GN=TNNC2 PE=1 SV=2			X				
Fibrinogen alpha chain OS=Sus scrofa GN=FGA PE=4 SV=1			X		X		
60S acidic ribosomal protein P2 OS=Sus scrofa GN=RPLP2 PE=3 SV=1				X			
c_Keratin14				X		X	X
Histone H4 OS=Sus scrofa PE=1 SV=2				X			
Histone H2A OS=Sus scrofa GN=HIST2H2AC PE=3 SV=1				X			
Myosin light polypeptide 6 OS=Sus scrofa GN=MYL6 PE=1 SV=2				X			
40S ribosomal protein S3 OS=Sus scrofa GN=RPS3 PE=2 SV=1				X			
60S ribosomal protein L18 (Fragment) OS=Sus scrofa PE=3 SV=1				X			
40S ribosomal protein S20 OS=Sus scrofa GN=RPS20 PE=3 SV=1				X			
40S ribosomal protein S13 (Fragment) OS=Sus scrofa GN=RPS13 PE=2 SV=2				X			
Histone H3 (Fragment) OS=Sus scrofa GN=LOC100627410 PE=3 SV=1				X			

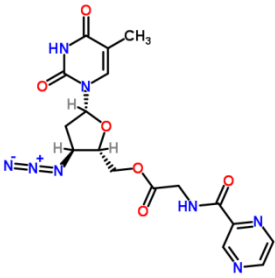
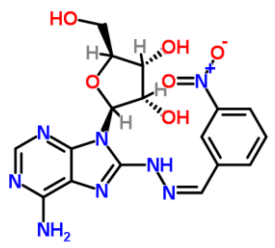
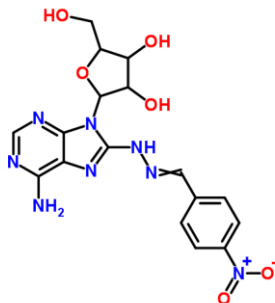
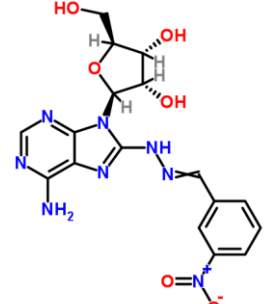
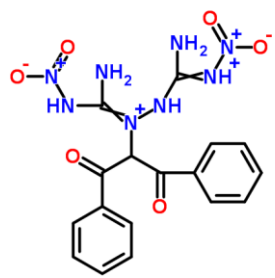
Peptide description	Peak I				Peak II	Peak III	Peak IV
	C9	C10	C11	C12	D10	D5	E9
Prelamin-A/C OS=Sus scrofa GN=LMNA PE=2 SV=1				X			
Elongation factor 1-alpha OS=Sus scrofa GN=EEF1A PE=2 SV=1				X			
GNAS complex locus OS=Sus scrofa GN=GNAS PE=2 SV=1				X			
Triosephosphate isomerase OS=Sus scrofa GN=TPI1 PE=2 SV=1				X			
40S ribosomal protein S12 OS=Sus scrofa GN=RPS12 PE=2 SV=2				X			
40S ribosomal protein S8 (Fragment) OS=Sus scrofa GN=LOC100516861 PE=3 SV=2				X			
Profilin OS=Sus scrofa GN=PFN1 PE=3 SV=1				X			
GTP-binding nuclear protein Ran OS=Sus scrofa GN=RAN PE=2 SV=2				X			
60S ribosomal protein L22 OS=Sus scrofa GN=RPL22 PE=2 SV=2				X			
Heat shock protein HSP 90-alpha OS=Sus scrofa GN=HSP90AA1 PE=2 SV=3				X			
Nucleoside diphosphate kinase B OS=Sus scrofa GN=NME2 PE=2 SV=1				X			
Phosphoglycerate kinase (Fragment) OS=Sus scrofa GN=PGK1 PE=3 SV=1				X		X	
Ubiquitin-conjugating enzyme E2 D2 (Fragment) OS=Sus scrofa GN=UBCH5B PE=3 SV=1				X			
Ras-related protein Rab-11A OS=Sus scrofa GN=RAB11A PE=2 SV=3				X			
Tubulin beta chain OS=Sus scrofa GN=TUBB PE=2 SV=1				X			
Beta-enolase OS=Sus scrofa GN=ENO3 PE=3 SV=1				X			
Cofilin-1 OS=Sus scrofa GN=CFL1 PE=1 SV=3				X			
Eukaryotic translation initiation factor 4A isoform 1 OS=Sus scrofa GN=EIF4A1 PE=2 SV=1				X			
60S ribosomal protein L35 OS=Sus scrofa GN=RPL35 PE=2 SV=3				X			
Peroxiredoxin-6 OS=Sus scrofa GN=PRDX6 PE=2 SV=3				X			
Annexin OS=Sus scrofa GN=ANXA1 PE=3 SV=1				X			
Histone H2B (Fragment) OS=Sus scrofa PE=3 SV=2				X			
Annexin OS=Sus scrofa GN=ANXA8 PE=3 SV=1				X			
60S ribosomal protein L13 OS=Sus scrofa GN=RPL13 PE=3 SV=1				X			
60S ribosomal protein L6 OS=Sus scrofa GN=RPL6 PE=2 SV=3				X			
ATP synthase subunit alpha (Fragment) OS=Sus scrofa GN=ATP5A1 PE=3 SV=1				X			
Elongation factor 1-beta OS=Sus scrofa GN=EEF1B PE=1 SV=1				X			
60S ribosomal protein L27 OS=Sus scrofa GN=RPL27 PE=2 SV=1				X			
40S ribosomal protein S19 (Fragment) OS=Sus scrofa GN=RPS19 PE=2 SV=3				X			
Glyceraldehyde-3-phosphate dehydrogenase (Fragment) OS=Sus scrofa GN=GAPDH PE=2 SV=1				X			
Fructose-bisphosphate aldolase OS=Sus scrofa GN=ALDOC PE=2 SV=2				X			
Apolipoprotein A-I OS=Sus scrofa GN=APOA1 PE=1 SV=4				X	X		
Annexin OS=Sus scrofa GN=ANXA4 PE=3 SV=1				X			
Moesin OS=Sus scrofa GN=MSN PE=4 SV=1				X			
Ribosomal protein L15 OS=Sus scrofa GN=RPL15 PE=2 SV=1				X			
60S ribosomal protein L14 OS=Sus scrofa GN=RPL14 PE=2 SV=1				X			
Histone H1.3-like protein OS=Sus scrofa GN=HIST1H1D PE=2 SV=1				X			
60S ribosomal protein L13a OS=Sus scrofa GN=RPL13A PE=2 SV=1				X			
Epidermal fatty acid-binding protein OS=Sus scrofa GN=FABP5 PE=2 SV=1				X			
60S ribosomal protein L11 OS=Sus scrofa GN=RPL11 PE=3 SV=1				X			
D-3-phosphoglycerate dehydrogenase OS=Sus scrofa GN=PHGDH PE=3 SV=1				X			

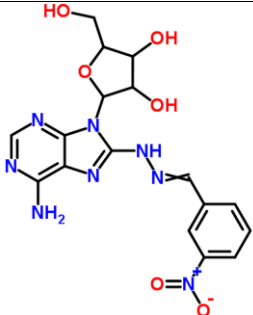
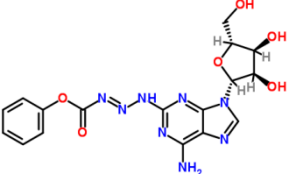
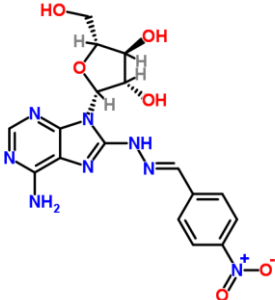
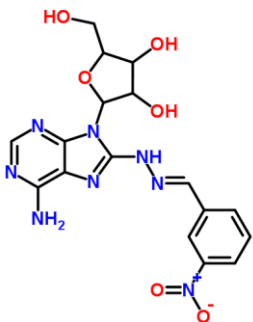
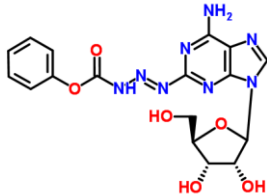
Peptide description	Peak I				Peak II	Peak III	Peak IV
	C9	C10	C11	C12	D10	D5	E9
40S ribosomal protein S6 (Fragment) OS=Sus scrofa GN=RPS6 PE=3 SV=2				X			
Elongation factor 1-gamma (Fragment) OS=Sus scrofa GN=EEF1G PE=2 SV=2				X			
Glycolipid transfer protein OS=Sus scrofa GN=GLTP PE=4 SV=2				X			
Guanine nucleotide-binding protein subunit beta-2-like 1 OS=Sus scrofa GN=GNB2L1 PE=4 SV=1				X			
Histidine ammonia-lyase OS=Sus scrofa GN=LOC100154617 PE=3 SV=2				X			
Galectin OS=Sus scrofa GN=LGALS3 PE=2 SV=1				X			
ATP synthase subunit beta OS=Sus scrofa GN=ATP5B PE=3 SV=1				X			
Voltage-dependent anion-selective channel protein 2 OS=Sus scrofa GN=VDAC2 PE=2 SV=1				X			
Vimentin OS=Sus scrofa GN=VIM PE=1 SV=2				X			
Serine/arginine-rich splicing factor 1 OS=Sus scrofa GN=SRSF1 PE=2 SV=3				X			
Transitional endoplasmic reticulum ATPase OS=Sus scrofa GN=VCP PE=1 SV=5				X			
78 kDa glucose-regulated protein OS=Sus scrofa GN=HSPA5 PE=3 SV=2				X			
Transaldolase (Fragment) OS=Sus scrofa GN=TALDO1 PE=3 SV=2				X			
60S acidic ribosomal protein P0 OS=Sus scrofa GN=RPLP0 PE=2 SV=2				X			
6-phosphogluconate dehydrogenase, decarboxylating (Fragment) OS=Sus scrofa GN=PGD PE=2 SV=3				X			
Glucose-6-phosphate isomerase OS=Sus scrofa GN=GPI PE=2 SV=2				X			
ROA1 OS=Sus scrofa GN=HNRNPA1 PE=2 SV=1				X			
60S ribosomal protein L3 OS=Sus scrofa GN=RPL3 PE=2 SV=2				X			
Rab GDP dissociation inhibitor beta OS=Sus scrofa GN=GDI2 PE=2 SV=1				X			
Inter-alpha-trypsin inhibitor heavy chain H4 OS=Sus scrofa GN=ITI4 PE=1 SV=1				X			
Putative trypsinogen OS=Sus scrofa GN=try PE=3 SV=1							X
contaminant_CASB_BOVIN							X

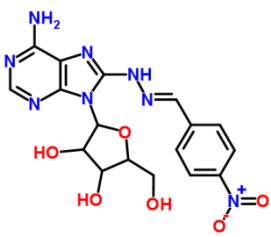
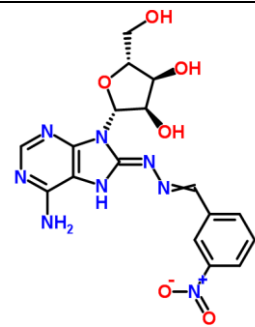
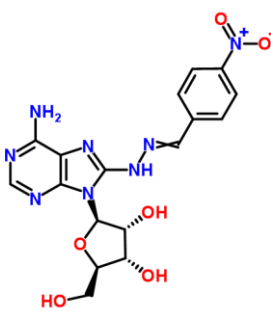
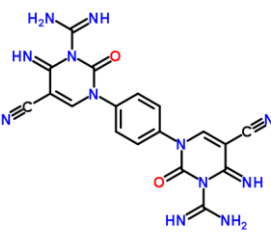
Appendix C

– Selected ChemSpider hits for the m/z 431 ion

Ion formula #	Structure	Name	ChemSpider ID
3		9-[(2R,3R,4S,5R)-3,4-dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl]-1-[(2R,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydropyran-2-yl]purin-6-one	8940374
		3-[3-(Carboxymethoxy)-5-methyl-1,2-oxazol-4-yl]alanine - 3-(5-methyl-3-oxo-2,3-dihydro-1,2-oxazol-4-yl)alanine (1:1)	25031621
4		8-[(2E)-2-(4-Nitrobenzylidene)hydrazino]adenosine	17515363
		8-[(2E)-2-(3-Nitrobenzylidene)hydrazino]adenosine	17463536

	3'-Azido-3'-deoxy-5'-O-[N-(2-pyrazinylcarbonyl)glycyl]thymidine	9692373
	8-[(2Z)-2-(3-Nitrobenzylidene)hydrazino]-9-(β-L-ribofuranosyl)-9H-purin-6-amine	5363428
	8-[2-(4-Nitrobenzylidene)hydrazino]-9-pentofuranosyl-9H-purin-6-amine	3466548
	8-[2-(3-Nitrobenzylidene)hydrazino]-9-(β-L-ribofuranosyl)-9H-purin-6-amine	5363427
	1-[Amino(nitroamino)methylene]-2-[amino(nitroiminio)methyl]-1-(1,3-dioxo-1,3-diphenyl-2-propenyl)hydrazinium	3302814

	8-[2-(3-Nitrobenzylidene)hydrazino]-9-pentofuranosyl-9H-purin-6-amine	2970405
	2-[(2E)-3-(Phenoxycarbonyl)-2-triazen-1-yl]adenosine	9070535
	9-(β-D-Arabinofuranosyl)-8-[(2E)-2-(4-nitrobenzylidene)hydrazino]-9H-purin-6-amine	17540217
	8-[(2E)-2-(3-Nitrobenzylidene)hydrazino]-9-pentofuranosyl-9H-purin-6-amine	17540715
	(2E)-2-[(2E)-3-(Phenoxycarbonyl)-2-triazen-1-ylidene]-9-(D-ribofuranosyl)-2,9-dihydro-1H-purin-6-amine	24653191

		8-[(2E)-2-(4-Nitrobenzylidene)hydrazino]-9-pentofuranosyl-9H-purin-6-amine	29770815
		8-[(3-Nitrobenzylidene)hydrazono]-8-hydroadenosine	35319419
		8-[2-(4-Nitrobenzylidene)hydrazino]adenosine	35360694
6		(6E,6'E)-3,3'-(1,4-Phenylene)bis(5-cyano-6-imino-2-oxo-3,6-dihydro-1(2H)-pyrimidinecarboximidamide)	8608245

Paper I

Pedersen, S. T., Baron, C. P., De Gobba, C., Duedahl-Olesen, L., Koch, A. G. & Jessen, F. Antioxidative and antimicrobial activity of extracts from nitrite-cured cooked pork products. *Manuscript close to submission.*

1 **Title:** Antioxidative and antimicrobial activity of extracts from nitrite-cured cooked pork products

2 **Author:** Sabine Tauber Pedersen^{a*}, Caroline P. Baron^a, Cristian De Gobba^b, Lene Duedahl-
3 Olesen^a, Anette Granly Koch^c & Flemming Jessen^a.

4 ^aNational Food Institute, Technical University of Denmark, Søltofts Plads Building 227, DK-2800
5 Kongens Lyngby, Denmark

6 ^bUniversity of Copenhagen, Department of Food Science, Ingredient and Dairy Technology,
7 Rolighedsvej 26, DK-1958 Frederiksberg C, Denmark

8 ^cDanish Meat Research Institute, Gregersensvej 9, DK-2630 Taastrup, Denmark

9

10 **Running title:** Antioxidative and antimicrobial activity of extracts from cured hams

11

12 ***Corresponding author:**

13 Sabine Tauber Pedersen

14 Postal address:

15 National Food Institute

16 Søltofts Plads Building 227, 1st floor, room 142

17 DK-2800 Kgs. Lyngby

18 E-mail & phone number:

19 stape@food.dtu.dk

20 +45 45252691

21

22 **Declarations of interest:** none

23 **Abstract**

24 The role of nitrite for oxidative and microbial stability of cured meats has been extensively studied
 25 yet focused mainly on nitrite itself. This study aimed to broaden the understanding of the role of
 26 nitrite in cooked cured hams, by investigating *in vitro* antioxidative and antimicrobial properties.
 27 Three nitrite-cured cooked hams were compared to a non-nitrite-cured cooked ham. Aqueous
 28 ≤ 10 kDa extracts of the nitrite-cured hams demonstrated higher *in vitro* reducing power and ABTS
 29 radical scavenging activities than the uncured, and activities appeared to correlate with residual
 30 nitrite/ascorbate. Iron chelating activity was found in two nitrite-cured hams and in the uncured. 3-
 31 nitrotyrosine was neither detected in hams nor extracts. Antioxidant activity may be linked to
 32 histidine-content. None of the extracts inhibited growth of *Listeria monocytogenes* or *Salmonella*
 33 *typhimurium*. In conclusion, the increased antioxidant activity obtained by nitrite-curing is not
 34 solely due to residual ascorbate but also to compounds formed during the processing.

35

36 **Keywords:** ham, nitrite-curing, antioxidant, *in vitro* assays, 3-nitrotyrosine, antimicrobial

37

38

39

40

41

42

43

44 **1 Introduction**

45 Salting of meat is an ancient preservation technique. By binding water, the salt decreases the water
46 activity below levels required for microbial growth and thereby protects meat and fish from
47 spoilage. Through times it became evident that salts containing saltpetre (KNO_3) contributed with
48 additional attributes in conservation, taste and colour, compared to pure salt (Binkerd & Kolari,
49 1975). Today meat curing consists of adding salt containing nitrite or nitrate and other ingredients
50 such as sugar and spices to fresh meat to create a unique flavour and colour. Curing is generally
51 done by either dry curing or wet curing or a combination. The resulting products may be intended
52 for consumption in raw state or may be submitted to further downstream processing like boiling and
53 smoking leading to an immense variety of processed cured meat products. Yet, the primary purpose
54 is still to prolong shelf-life by preventing microbiological spoilage, growth of pathogens and
55 oxidative degradation.

56 Indeed, nitrite is a strong oxidant but in combination with common meat additives like ascorbate
57 and salt they collectively possess the ability to reduce lipid oxidation (Honikel, 2008). For example,
58 Morrissey & Tichivangana (1985) found that levels as low as 20ppm nitrite was sufficient to
59 significantly inhibit lipid oxidation in fish, chicken, pork, and beef systems also when adding pro-
60 oxidants. Also, Mac Donald, Gray, Kakuda & Lee (1980) found a significant reduction in
61 Thiobarbituric acid (TBA) values in pork when treated with any level of nitrite (50, 200, and
62 500ppm) compared to a non-nitrite treatment.

63 During processed meat manufacturing, a complex web of reaction is occurring between reactive
64 nitrite (species), meat constituents and additives and at the end of the heat treatment residual nitrite
65 is reduced to less than one third of the originally added amounts (Honikel, 2008). Furthermore,
66 residual nitrite declines even further during cold storage, occasionally to non-detectable levels
67 (Honikel, 2008).

68 The main reactivity of nitrite is exerted through nitric oxide (NO) which reacts with a wide variety
69 of molecules such as myoglobin, ascorbic acid or amino acids and other proteins (Honikel, 2008).

70 The most studied reaction related to nitrite curing is the reaction of NO and myoglobin.

71 Yet, Tricker & Kubacki (1992) summarizes that only 5-15% of added nitrite is associated with
72 myoglobin while 5-10% is recovered as nitrite and 1-10% is oxidized to nitrate. Furthermore 1-5%
73 is thought to be lost as gasses and 1-5% is believed to be associated with lipids. Of the remaining
74 portion 5-15% may react with sulfhydryl groups, mostly in peptides and free amino acids, while as
75 much as 20-30% will bind to other proteins (Tricker & Kubacki, 1992).

76 Nitrite induced modification on proteins, peptides and amino acids mainly entail the addition of
77 NO. Examples of such are the S-nitrosylation of the cysteine thiol, the C-nitrosation of tyrosine, or
78 the N-nitrosation of amines among others leading to formation of nitrosamines. With the exemption
79 of N-nitrosamines, the existence and understanding of the properties of these modified peptides are
80 to our knowledge relatively limited in regard to food. However, amino acids and small proteins
81 modified by nitrite like S-nitrosocystein, and S-nitrosoglutathione have previously been found to
82 possess antioxidant properties (Kanner, 1979) (Chiueh & Rauhala, 1999) (Morrissey &
83 Tichivangana, 1985).

84 Within the last few decades, increasing research in antioxidant and antimicrobial peptides from
85 different food and food related matrixes, has led to the discovery of the naturally occurring
86 antioxidant dipeptides, carnosine and anserine. But also small active peptides in hydrolysates of
87 different animal tissue and animal based products has been identified (Review: Di Bernardini *et al.*,
88 2011a) (Gil-Agustí, Esteve-Romero & Carda-Broch, 2008). However, to our knowledge, the
89 existence and extent of antioxidant activities from nitrite wet-cured cooked ham, beyond the direct
90 impact of nitrite, has not been fully investigated.

Thus, it can be hypothesised that compounds with antioxidative and antimicrobial properties are formed during nitrite curing and that these compounds might contribute to the mechanisms by which nitrite exerts its preservation properties. Furthermore, it can be speculated that some of these compounds may be low molecular weight compounds such as peptides that to some extent have been nitrosylated/nitrosated by nitrite.

Thus, we set out to investigate the nature of the antioxidative and antimicrobial activities generated during nitrite-curing

2 Material and methods

2.1 Materials

Ascorbic acid, sodium nitrite, 3-nitro-L-tyrosine, 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-4',4''-disulfonic acid sodium salt (ferrozine) and 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), were from Sigma-Aldrich (St. Louis, MO, USA).

Ethylenediaminetetraacetic acid disodium dihydrate (EDTA) was from Merck Millipore (Billerica, MA, USA) and (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) from Fluka Chemika (Deisenhofen, Germany). All remaining chemicals and reagents used were of analytical grade and acquired from VWR International (Radnor, PA, USA), Merck Millipore, Sigma-Aldrich or Fluka Chemika.

2.2 Hams

The sample material comprised three different types of commercial nitrite-cured cooked pork products: a sandwich ham (SH), a boiled dinner ham (DH) and a smoked pork saddle (SPS). Pictures of the three products can be found in Figure 1. The sandwich ham was a ready-to-eat luncheon meat product belonging to the category of restructured meat products where smaller

114 pieces of meat were subjected to curing brine prior to boiling stuffed into a plastic casing. The
115 remaining two products were whole-muscle products most likely injected with curing brine prior to
116 boiling and smoking (only the pork saddle was smoked). The exact manufacturing procedures were
117 undisclosed by the producers yet the following details were provided: SH was added 60ppm nitrite
118 and unknown amounts of sodium ascorbate (E301) while DH and SPS was both added 120ppm
119 sodium nitrite (E250) and unknown amounts of sodium ascorbate (E301). Furthermore, SPS was
120 added phosphates (E450, E451). The sandwich ham, SH, was delivered from the manufacturer in
121 bulk. The whole-muscle products, DH and SPS, were supplied from the manufacturer in consumer
122 packaging. For the four days from production to arrival at the laboratory, the vacuum-packed
123 samples were stored at 5-8°C and afterwards moved to -80°C until further analysis.

124 The samples were tested without any further heat-treatment. Prior to sampling for the individual
125 analytical methods a larger piece of the ham, of approximately 100g, were minced to ensure a
126 homogenous sample. The remaining skin and the excess subcutaneous fat on the dinner ham (Figure
127 1) were trimmed before mincing. The smoke-coloured surface of the pork saddle (Figure 1) was not
128 removed as it is expected to be consumed by the majority of Scandinavian consumers.

129 A reference ham prepared without added nitrite or ascorbic acid was included. The reference ham
130 was a boiled restructured ham made from pork, water, salt, pork skin protein, phosphates, potato
131 starch and dextrose.

132

133 *2.3 Proximate composition analysis*

134 *2.3.1 Nitrite content*

135 Extraction and measurement of nitrite in the cured and uncured cooked pork products was done
136 according to Hermann, Duedahl-Olesen & Granby (2015). In short, the detection was carried out

137 using ion chromatography coupled to UV detection at 225nm (HPLC and Diode Array Detector,
138 series1100, Agilent Technologies, Santa Clara, CA, USA) with separation on a Dionex IonPac™
139 AS11 RFICTM 250 x 4mm (Lot # 011-33-189A, product # 044076, serial #020071, Thermo
140 Scientific, Waltham, MA, USA). The samples were analysed in a random order. External
141 calibration was performed in the range 0.25-100µg/mL NaNO₂. As the nature of the added nitrite
142 was unknown in SH all results are presented as ppm nitrite-ion by the relationship 1g nitrite ion =
143 1.50g NaNO₂.

144 2.3.2 Ascorbic acid content

145 Residual ascorbic acid in the hams were determined using an Agilent 1100 series HPLC using a
146 G1315A DAD detector set at 254.4nm (Agilent Technology, Waldbronn, Germany).
147 Ascorbic acid was extracted by leaving 2.5g of minced ham and 10mL of 5%(W/V) meta-
148 phosphoric acid, added 0.5mL of 0.1mg/mL EDTA solution, to stir for 2min at room temperature
149 protected from light. MilliQ was added to a total volume of 25mL. The mixture was centrifuged at
150 3000rpm for 5min and filtered through a Munktell quantitative filter paper grade 00H and re-filtered
151 through a 0.2µm syringe filter directly into an amber HPLC vial. Twenty µL was injected and
152 separated on a Prodigy 5u ODS3 100Å 250x4.6mm column (Phenomenex®, Torrance, CA, USA)
153 and eluted at a flow rate of 0.7mL/min. Mobile phase A was water adjusted to pH 3 and B was
154 methanol running a gradient of 5% B for the first 23min followed by a 10sec linear increase to
155 100% B which were kept for 4min and 50sec before again decreasing to 5% B in 10sec and kept at
156 this level until the end of the run (total of 35min). Quantification was done using an external
157 calibration curve of L-ascorbic acid dissolved in 1%(W/V) meta-phosphoric acid.

158 2.3.3 Amino acid profile (total and free)

159 The total and free amino acid content of the hams was determined according to the procedure
160 described by Farvin, Baron, Nielsen, Otte & Jacobsen (2010) with slight modifications. In short, the

total amino acid content was analysed by subjecting approximately 30mg of ham to microwave-assisted acidic hydrolysis (6M HCl) for 1 hour at 110°C and 500W followed by 10min of cooling. The samples were diluted with 1M NaCO₃ and filtered through a 0.2µm syringe filter (Q-max PTFE, Ø13mm, Frisenette ApS, Knebel, Denmark) before derivatisation using the EZ:FaastTM Amino Acid Analysis kit from Phenomenex® (Torrance, CA, USA). The derivatised samples were analysed by LC-(APCI)-MS (Agilent 1100, Agilent Technology). Separation took place on a reversed phase column (EZ:FaastTM AAA-MS 250 x 3.0mm, Phenomenex®, Torrance, CA, USA) running a gradient (mobile phase A was water and B was methanol, both containing 10 mM ammonium formate) at 0.5mL/min. Total runtime was 26min.

Prior to derivation of free amino acid using the EZ:FaastTM Amino Acid Analysis kit, 250µL of TCA was added to 100µg of ham and left over night at 4°C, centrifuged at 12,000rpm for 3min and 100µL hereof was mixed with 50µL of 1M NaCO₃ which was then analysed as described above.

2.4 Extraction of low molecular weight compounds

Water soluble compounds were extracted from the hams by homogenising 94.9-101.5g of minced ham in approximately 400mL of deionised water, dH₂O, for 1min with an Ultra Turrax (T 25 Basic ULTRA-TURRAX® mounted with S25N -18G Dispersing element, IKA®-WERKE GmbH & Co. KG, Staufen, Germany) at 13,500rpm and subsequently adding up to a total volume 1.5L. Extraction from the reference ham was done in the same manner however at a 1:10 ratio. An aqueous ≤10kDa extract was collected by submerging five times 10cm dialysis membrane tubes (Spectra/Por 6, MWCO 10,000Da, Spectrum Lab. Inc, CA, USA) containing 16.5mL dH₂O (corresponding to 50% membrane volume) into the meat/water homogenate. After 24 hours of magnetic stirring at 5°C, the dialysate in the 5 membranes were pooled and stored at -80°C until further analysis. Later the protein content was determined using the PierceTM Modified Lowry Protein Assay Kit in microplate format according to manufactures description (Thermo Scientific,

PierceTM) using Bovine Serum Albumin (BSA) (Ampules, 2 mg/ml) supplied with the kit as an external standard.

2.5 Antioxidant activity

Potential antioxidant activities of the aqueous ≤ 10 kDa extract were analyzed using three *in vitro* assays measuring iron chelation activity, reducing power and ABTS-radical scavenging activity. In order to investigate the concentration dependency of the antioxidant activities, the crude undiluted ≤ 10 kDa extract were analyzed followed by sequential threefold dilutions. The assays were conducted in triplicate and all measurements were performed using a spectrophotometer (Synergy 2 Multi-Mode Microplate Reader, BioTek® Instruments, Inc., Vermont, USA). The extracts were thawed at 5°C, over night.

Iron chelating activity was assayed by the microtiter plate format method described by Hermund *et al.* (2015). EDTA (0.5 mM) was used as a positive control and was subjected to the same threefold dilutions as the extract samples. dH₂O was utilized as blank. The iron chelating activity (%) is defined and calculated as percentage of inhibited colour formation/relative decrease in absorbance (562 nm):

$$Fe^{2+} \text{ chelating activity (\%)} = \frac{Abs_{blank} - Abs_{sample}}{Abs_{blank}} \cdot 100\%$$

The reducing power was determined according to the procedure described by Oyaizu (1986) with some modifications and adjusted to microtiter plate detection. First, equal parts of ≤ 10 kDa extract sample (and dilutions), 0.2 M phosphate buffer (pH 6.6) and 1% potassium ferricyanide were mixed and incubated for 20 min at 50°C. Subsequently, 1 part of 10% TCA was also added. Then, 100 μ L of the sample mixture was mixed with 100 μ L dH₂O and 20 μ L of 0.1% ferric chloride, and incubated 10 min at room temperature. The absorbance was measured at 700 nm. Ascorbic acid

(0.5mM) was used as positive control and was subjected to the same threefold dilution as the extract samples and dH₂O was used as blank. Results are expressed as

$$OD_{700} = Abs_{700nm}(Sample) - Abs_{700nm}(blank)$$

The ABTS radical scavenging activity was determined as described by Gringer, Osman, Nielsen, Undeland & Baron (2014). Trolox (2.5mM, in 96% ethanol) was used as a positive control and was subjected to the same threefold dilutions as the samples. Ethanol (96%) and dH₂O was included as blank for the positive control and extract sample, respectively. The ABTS radical scavenging activity (%) is expressed as the relative decrease in absorbance (734nm) as for iron chelating activity.

2.6 Antimicrobial activity

Antimicrobial activity of the cured ham aqueous ≤10kDa extracts and the supernatant of the meat/water homogenate were tested with an inoculation culture consisting of a mixture of *Listeria monocytogenes* (DMRICC 3042) and *Salmonella typhimurium* (DMRICC 4984). A small quantity of the purified cultures were each transferred to 10mL Brain-heart infusion (BHI) broth (Oxoid CM1135, United Kingdom) and incubated for 20 ± 2 hours at 37°C. These were afterwards diluted to 10⁻⁶ and mixed 1:1 (V/V) to constitute the inoculation culture.

Diluted (1:5 and 1:25) and undiluted extracts were mixed 1:1 with BHI broth and inoculated with the inoculation culture. The initial concentration of each bacteria culture in the inoculated samples was 44 cfu/ml ~ 1.6 log cfu/ml. A sample substituting extract with FKP (0.85% NaCl and 0.1% pepton (Merck 1.07214)) was included as a control. The inoculated extract samples were incubated at 17 ± 1°C and samples were taken after 0, 24, 48 and 72 hours. The total bacterial count was determined on Oxford agar (oxoid CM 0856, United Kingdom) and SSI Enteric agar (34121, Statens Serum Institute, Copenhagen Denmark) after 24-48 hours of incubation at 37°C.

2.7 3-Nitrotyrosine on LC-MS

The content of 3-nitrotyrosine (3-NT) was determined in both ≤ 10 kDa extracts and hams following acidic hydrolysis in 6M HCl. 0.5 mL of extract or 3-NT standard solutions were mixed 1:1 (V/V) with HCl while approximately 30 mg of freshly minced ham were added 1 mL of HCl. The mixtures were overlaid with nitrogen, closed and hydrolysed at 110°C. After 24 hours the samples were left to cool protected from light. Extract samples and external standards were transferred directly to HPLC vials while the hams samples were filtered through a 0.2 μ m syringe filter (Q-max PTFE, Ø13 mm, Frisette ApS) into the HPLC vial. 3-NT was quantified by LC-MS/MS using an UHPLC+ Ultimate 3000, mounted with a C18 column (Aeris Peptide XB-C18, 150 x 2.1 mm, 1.7 μ m, 100 Å, 40°C) coupled with a Q Exactive Biotech mass spectrometer (both ThermoFisher Scientific). Buffer A was 0.1% formic acid (FA) in water, and buffer B was 0.1% FA in 80% acetonitrile. 10 μ L of sample were injected. The flowrate was 0.25 mL/min and the gradient consisted of 100% A for 5 min, followed by a linear increase from 0% to 25% B in 15 min. 3-NT was detected and quantified in PRM mode using 227.06 m/z as parent ion with a resolution of 17500 and AGC target of $2e^5$, max IT 64 ms. The reporter ion used for quantification was 181.06 m/z. 3-NT solutions of 0.5-500 ng/mL (after hydrolysis) were used for external calibration.

2.8 Statistical analysis

Measurements were carried out in triplicate (unless stated otherwise). Results are presented as average values \pm the standard deviation. All statistical analyses were conducted using the GraphPad Prism® software Ver. 4.03 or R version 3.3.0 with $P < 0.05$. Results were compared using one-way ANOVA and t-test with Tukeys posttest.

252 **3 Results and discussion**

253 *3.1 Antioxidative activity*

254 The current study set out to investigate whether antioxidant and antimicrobial activity could be
255 identified in aqueous ≤ 10 kDa extracts of three different nitrite-cured cooked pork products. Using
256 *in vitro* assays for iron chelating activity, reducing power and ABTS radical scavenging activity,
257 antioxidant activity was demonstrated for all aqueous ≤ 10 kDa extracts in all assays with a single
258 exception (Figure 2).

259 The ≤ 10 kDa extracts of SH, SPS and the non-cured reference ham possessed a maximal iron
260 chelating activity of 70%, 62% and 90%, respectively, at the highest tested concentrations, whereas
261 the extract from DH showed no activity (Figure 2A). In comparison the positive control in the
262 tested concentration (0.5mM EDTA) demonstrated 95-99% iron chelating activity (data not shown).

263 The antioxidant activities may be viewed as the initial linear activity increase per mg ham and is
264 presented in Table 1. From the curves in Figure 2A the reference ham seems to have the highest
265 initial linear iron chelating activity increase which from the first 2-3 data points may be calculated
266 to 577 ± 187 % iron chelating activity/mg ham. In comparison, SH and SPS had initial linear iron
267 chelating activity increase of 54 ± 4 and 24 ± 1 % iron chelating activity/mg ham.

268 From the current results iron chelating activity did not appear to be connected with nitrite curing as
269 the reference ham, without added nitrite, showed iron chelating activity and an initial linear iron
270 chelating activity increase much higher than the cured SPS and SH while, as already mentioned, the
271 cured DH showed no activity.

272 The reducing power of the aqueous ≤ 10 kDa extracts are presented in Figure 2B. All extract
273 displayed lower activity than the positive control, ascorbic acid (data not shown). SPS showed the
274 highest activity with an initial linear activity increase (Table 1), which was about 4 times higher

275 than the initial linear activity increase of the reference ham, showing the lowest reducing power of
276 all the extracts. The DH extract again showed the lowest activity of the three nitrite-cured hams.

277 All ≤ 10 kDa extracts, including the reference, in crude form showed similar maximal ABTS radical
278 scavenging activities of 90-92% (Figure 2C), which was at the same level as the positive control,
279 trolox (data not shown). Compared to the reference ham, the initial linear radical scavenging
280 activity increase of SPS was approximately 2.5 times higher (Table 1).

281 As oppose to iron chelating both reducing power and ABTS radical scavenging properties seem to
282 be connected with curing by way of all the nitrite-cured ham extracts displaying higher antioxidant
283 properties than the reference. However, the reducing power and ABTS radical scavenging
284 properties did not correlate with added nitrite/ascorbic acid but rather with residual nitrite/ascorbic
285 acid (Table 2). Although, this indicates a correlation between residual nitrite/ascorbic acid and
286 ABTS radical scavenging activity.

287 High diversity in laboratory and data treatment methodologies makes it highly problematic to
288 compare current findings with previously reported data. However, antioxidant peptides have been
289 identified and purified from different dry-cured hams (Xing *et al.*, 2016)(Escudero, Mora, Fraser,
290 Aristoy & Toldrá, 2013). Mora, Escudero, Fraser, Aristoy & Toldrá (2014) identified peptides of
291 400-2500 Da and tested a selected few purified peptides for DPPH radical scavenging activity and
292 reducing power. ABTS and DPPH radical scavenging assays both describe a sample's ability to
293 scavenging radicals but they are not directly comparable (de Casto & Sato, 2015). However, the
294 peptides, purified by Mora *et al.* (2014), which demonstrated the highest reducing power had a
295 reducing power absorbance (corresponding to OD₇₀₀ in the current work) of approximately 1.5 at
296 0.2 mg/mL while the majority of the purified peptides were below 0.5 at the same concentration.
297 Also Escudero *et al.* (2013) found even lower reducing power absorbance for their purified peptides

at 0.2mg/mL. In the current study the total protein concentration was approximately 0.2mg/mL in all extracts (SH~0.23mg/mL, DH~0.22mg/mL, SPS~0.19mg/mL and reference~0.23mg/mL), yet they still gave rise to a reducing power antioxidant activity of 0.25-0.5. Thus, if the observed antioxidant capacities could be related to a single or a few peptides these may be more powerful reducing antioxidant on their own than the majority of the once tested in the referred studies. Otherwise, the currently demonstrated reducing power activity may be caused by strong synergetic effects of protein and non-protein, anti- and pro-oxidant compounds in the extracts.

Residual nitrite and small active nitrite-derived compounds e.g. NO, N₂O₃ and HNO₂ can freely pass the dialysis membrane and the presence of these might interfere with the assays because of their ability to prevent oxidation of lipids (Skibsted, 2011). The residual nitrite (Table 2) in itself was not considered an issue however, it cannot be ruled out that the nitrite may function as a minute reservoir for NO or other nitrosating or antioxidant agents especially in the presence of a reducing agent such as ascorbic acid (Table 2). Many mechanisms on how nitrite exerts its antioxidant capabilities have been suggested through the past decades. The most predominant (no rank order) constitutes (I) oxygen sequestering by oxidation of NO to NO₂, (II) the formation of a stable complex between heme-iron and nitrite preventing release of iron, (III) stabilisation of unsaturated fatty acids, (IV) formation of nitroso- and nitrosyl compounds having antioxidant properties, like S-nitrosocystein and (V) chelating of iron and free radicals (Honikel, 2008)(Freyblier *et al.*, 1993) (Skibsted, 2011). Especially the last mechanism of nitrite will have a direct impact on the used antioxidant assay. Consequently, the detected antioxidant activity of the extracts would appear higher than if the activity was only attributed to by other unknown extract constituents. In high concentrations, nitrite behaves as a strong pro-oxidant. However, this effect requires free nitrite concentrations to exceed the levels found in commercially cured meat products (Mac Donald, Gray & Gibbins, 1980).

322 In addition to nitrite, (small free) ions (metal ions or salt), present in plenty in meat, are also capable
323 of passing the dialysis membrane and might therefore also interfere with the antioxidant assays. As
324 the iron chelating assay is based on the principle of neutralising added iron pro-oxidant, the
325 presence of additional ferrous ions would result in the nitrite-cured ham extracts appearing as less
326 potent antioxidant pools. In order to correct for this error, in future studies, it would be necessary to
327 include a control containing, ≤ 10 kDa extract samples and ferrozine but substituting FeCl_2 with
328 dH_2O . Furthermore, NaCl is often mentioned in regard to decreasing oxidative stability of muscle
329 foods. Some of the pro-oxidative mechanisms are related to the chloride ion altering iron's
330 reactivity and the sodium ion causing displacement of iron from macromolecules (Lee, Mei &
331 Decker, 1997). Both of which may have affected the iron chelating assay.

332 Sodium ascorbate is a common additive in nitrite-cured pork products and was also added to the
333 three nitrite-cured hams: SH, DH, and SPS. Sodium ascorbate is added for its curing accelerating,
334 nitrosamine reducing and antioxidant properties (Sebranek, 2009). The ascorbate ion functions as a
335 reducing agent and even though ascorbic acid is very light and heat sensitive residual amount was
336 expected to be present in the hams. Ascorbic acid would be able to pass the dialysis membrane and
337 thus result in the aqueous ≤ 10 kDa extracts exhibiting a higher reducing power activity. The order of
338 hams in regard to detected residual ascorbic acid ($\text{SPS} > \text{SH} > \text{DH} > \text{Reference}$) also matched the order
339 of the ham extracts in regard to reducing power (both the initial linear and at maximal tested extract
340 concentration). Thus, it must be assumed that residual ascorbic acid was a great contributor to the
341 detected reducing power yet a doubling in residual ascorbic acid did not correspond to a doubling in
342 reducing power. In addition, phosphates were also added to SPS. Some phosphates are known to
343 function as metal chelators and are as such expected to enhance the iron chelating activity signal.
344 However, no iron chelating was observed for the respective ≤ 10 kDa extract (Figure 2).

345

3.1.1 Amino acids composition of the hams

As indicated previously a potential contributor to the observed antioxidant activity could be peptides and amino acids originating from the hams. In the field of bioactive peptides a lot of effort has been invested in identifying amino acids of importance to activity. Either by isolation and further characterisation of active peptides or simply testing free amino acids in solution, it has become clear that some amino acids exhibit stronger, and different, antioxidant properties than others (Meucci & Mele, 1997)(Marcuse, 1962)(de Castro & Sato, 2015). Histidine, tyrosine, tryptophan, methionine, cysteine, phenylalanine and proline are some of the amino acids most frequently listed to have antioxidant activity either as free amino acids or embedded in a peptide (Di Bernardini *et al.*, 2011b) but also lysine, arginine and glutamic acid are ascribed as antioxidative (Broncano, Otte, Petró, Parra & Timon, 2012). Many of these have been observed to be of importance for metal chelation, including the acidic amino acids (Saiga, Tanabe & Nishimura 2003). However, the current results did not exactly reflect this effect as DH were found to contain the most protein (data not shown) and thus amino acids yet demonstrated the lowest iron chelating activity.

In order to evaluate the peptidic contribution to the measured antioxidant activity both the free and total amino acid profile was determined. The content of free amino acids was, as expected, very low: 3.14, 2.42 and 3.95mg amino acid/g DM in SH, DH and SPS respectively, and not significantly different between the three nitrite-cured hams. Overall the total amino acid profiles were not significantly different and all profiles were dominated by glutamic and aspartic acid (Figure 3) which matched the findings by Wilkinson, Lee, Purchas & Morel (2014) in both raw and cooked (60 and 75°C) pork muscle as well as data from the Danish food composition database (Frida Food Data - <http://frida.fooddata.dk/>). Glutamic acid constituted on average 14.6% in the nitrite-cured hams, and 15.4% and 14.4%, respectively for the two literature references. On the contrary the

nitrite-cured hams of the current study contained relatively more proline, histidine, serine and alanine and much less valine, isoleucine and threonine (Wilkinson *et al.*, 2014)(Frida Food Data, 2016). The relative higher content of the known antioxidative proline and histidine could have resulted in the tested commercial nitrite-cured hams exhibiting stronger antioxidative activity compared to other untested commercial nitrite-cured cooked pork products as well as the uncured reference ham.

Histidine has been found to possess antioxidant capacities as a radical scavenger, active oxygen quencher and metal ion chelator (Sun *et al.*, 2009). Thus, the relatively high content of histidine in the nitrite-cured hams (6.1-9.8%) could have contributed to the observed ABTS radical scavenging activity. Also the order of ABTS radical scavenging activity of extracts of the nitrite-cured hams and the uncured reference ham illustrated in Figure 2 fits with the relative histidine content.

Phenylalanine and in particular tyrosine are often mentioned in connection with electron donors and radical scavengers mainly due to their aromatic ring. Both amino acids were found to be present in the hams but it is not possible to speculate on their importance based on the current data however it cannot be ruled out that they might have contributed additively. Tryptophan has also been found to be of relevance in regard to (ABTS) radical scavenging (Nimalaratne, Lopes-Lutz, Schieber & Wu, 2011), and also reduction of iron and chelating of metals (de Castro & Sato, 2015) however, from the current data, no connection between the observed antioxidant activity and tryptophan content could be extrapolated, and the same goes for cysteine. However, this limitation of the data does not rule out that these amino acids might contribute to the antioxidant capacities.

3.2 3-Nitrotyrosine

Although sodium nitrite is highly researched for its importance in oxidative stability in meat newer research hypothesize that nitrosative stress induced on proteins by reactive nitrogen species e.g. peroxynitrite leads to the formation of specific compounds that might function as an alternative

394 marker for protein oxidation. Such a compound is the nitrosylated amino acid 3-nitrotyrosine. Most
395 research supports this hypothesis in a food context (Feng *et al.*, 2016) although Vossen & De Smet
396 (2015) based on their study rejected the hypothesis. In the current study 3-NT was found in neither
397 the ≤ 10 kDa fraction nor the meat of any of the nitrite-cured cooked hams and neither in the
398 reference with no added nitrite/ascorbate. If 3-NT can in fact be considered a proper marker for
399 protein oxidation the current results would indicate limited protein oxidation potentially due to the
400 presence of strong antioxidant compounds.

401 A basal level of 3-NT, most likely originating from physiological nitrosative stress, is usually
402 considered to be present in any muscle-based food (Villaverde, Parra & Estévez, 2014). With the
403 current method detecting 3-NT standard solution down to 0.5ng/mL it is odd that no 3-NT was
404 detected at all. Although increasing nitrite addition has been found to increase nitrosation degree of
405 tyrosine, ascorbate has also been found to inhibit 3-NT formation in a dose-dependent manner
406 (Villaverde *et al.*, 2014). Despite the limited information available about ascorbate addition to the
407 tested nitrite-cured hams this correlation might contribute to the explanation for the lack of basal
408 levels of 3-NT in the cured samples, yet it does not offer any explanation for the uncured reference
409 ham. Furthermore, heat treatment decreases the stability of nitro-amino acids and Feng *et al.*, 2015
410 also found this to be the case for 3-NT although they still retrieved some 3-NT in their heated
411 myofibrillar protein samples. 3-NT spiking of both ≤ 10 kDa extracts and hams in the current study
412 led to a recovery of approximately 80% in the extract yet no 3-NT was recovered from the ham
413 samples. This indicates that 3-NT formation may not just be inhibited in the complete hams, it
414 might even be degraded and that the degradation reaction(s) only occurs in the presences of
415 unknown compounds > 10 kDa.

416

417

3.3 Antimicrobial activity

Nitrite possesses antimicrobial activity against a variety of spoilage bacteria and foodborne pathogens common in meat products (Milkowski, Garg, Coughlin & Bryan, 2010). However, in the current study neither growth of *Listeria monocytogenes* nor *Salmonella typhimurium* were inhibited compared to the control by any of the nitrite-cured ham ≤ 10 kDa extracts, see Figure 4. Bacterial growth of either culture was very similar in all samples and followed the growth curve predicted using CombasePredictor (<http://www.combase.cc/>) (data not shown).

The supernatant of the meat/water homogenate used for producing the ≤ 10 kDa extracts neither demonstrated any antimicrobial activity in the tested concentrations.

The required amount of nitrite for sufficient antimicrobial efficacy seems to vary from product to product but much evidence points towards the in-going amount of nitrite rather than the residual amount of nitrite in the product being of importance (*C. botulinum*) (EFSA, 2003). Thus, it must be concluded that even though nitrite could freely pass the dialysis membrane it was present in too low concentration to be of any antimicrobial significance. To our knowledge antimicrobial compounds “natural” to nitrite-cured cooked pork has not been investigated. Yet, Castellano *et al.* (2016) identified ten anti-listerial peptides in dry cured hams, most likely generated in the hydrolysis occurring during ripening, while Wang (2003) extracted peptides of approximately 7kDa from porcine blood inhibiting growth of *Staphylococcus aureus* and *Escherichia coli*. However, if any of these or related compounds were at all present in the nitrite-cured hams, they have not been extracted in adequate concentration to prompt an antimicrobial response.

438

4 Conclusion

Effect of nitrite curing on antioxidant activity were evident in two out of three of the applied *in vitro* antioxidant activity assays performed on aqueous ≤ 10 kDa extracts of nitrite-cured cooked hams.

Nitrite curing increased or accelerated reducing power and ABTS radical scavenging activity compared to the non-cured reference. Although reducing power and ABTS radical scavenging activity correlated with residual rather than added nitrite/ascorbate, the increased antioxidative activity obtained by nitrite-curing is not due to nitrite itself and neither can it be assigned solely to residual ascorbate. In conclusion, compounds with relation to nitrite formed during curing and cooking must be important contributors to the demonstrated antioxidant activity. Such antioxidant compound might also inhibit protein oxidation although the lack of detectable 3-NT might also have other explanations such as degradation. The, differences in antioxidant activity may, for one, be linked to differences in histidine content. If nitrite curing also results in formation of antimicrobial compounds the activity from such compounds could not be affirmed in the investigated aqueous ≤ 10 kDa extracts.

Acknowledgements

This work was supported by a grant from Norma and Frode S. Jacobsens Fond. Thanks are expressed to Flemming Hansen for contributing in idea generation, sample material and data collection and for supervising in all microbiological matters.

Declarations of interest: none

6 References

Binkerd, E. F., & Kolari, O. E. (1975). The History and use of nitrate and nitrite in curing of meat. *Food Cosmetics Toxicology.*, 13, 655-661.

- 463 Broncano, J. M., Otte, J., Petrón, M. J., Parra, V., & Timón, M. L. (2012). Isolation and
464 identification of low molecular weight antioxidant compounds from fermented “chorizo” sausages.
465 *Meat Science*, 90, 494-501
- 466 Castellano, P., Mora, L., Escudero, E., Vignolo, G., Aznar, R., & Toldrá, F. (2016). Antilisterial
467 peptides from spanish dry-cured hams: Purification and identification. *Food Microbiology*, 59, 133-
468 141
- 469 Chiueh, C., & Rauhala, P. (1999). The Redox Pathway of S-Nitrosoglutathione, glutathione and
470 nitric oxide in cell to neuron communications. *Free Radical Research*, 31, 641-650
- 471 de Castro, R. J. S., & Sato, H. H.(2015). Biologically active peptides: Processes for their generation,
472 purification and identification and applications as natural additives in the food and pharmaceutical
473 industries. *Food Research International*, 74,185-198
- 474 Di Bernardini, R., Harnedy, P., Bolton, D., Kerry, J., O'Neill, E., Mullen, A. M., & Hayes, M.
475 (2011a). Antioxidant and antimicrobial peptidic hydrolysates from muscle protein sources and by-
476 products. *Food Chemistry*, 124, 1296-1307
- 477 Di Bernardini, R., Rai, D. K., Bolton, D., Kerry, J., O'Neill, E., Mullen A. M., Harnedy, P., &
478 Hayes, M. (2011b). Isolation, purification and characterization of antioxidant peptidic fractions
479 from a bovine liver sarcoplasmic protein thermolysin hydrolysate. *Peptides*, 32, 388-400
- 480 EFSA (2003) Opinion of the Scientific Panel on Biological Hazards on the request from the
481 Commission related to the effects of Nitrites/Nitrates on the Microbiological Safety of Meat
482 Products. *The EFSA Journal*, 14, 1-31
- 483 Escudero, E., Mora, L., Fraser, P. D., Aristoy, M.-C., & Toldrá, F. (2013). Identification of novel
484 antioxidant peptides generated in Spanish dry-cured ham. *Food Chemistry*, 138, 1282-1288

- 485 Farvin, K. H. S., Baron C. P., Nielsen, N. S., Otte, J., & Jacobsen, C. (2010). Antioxidant activity of
486 yoghurt peptides: Part 2 – Characterisation of peptide fractions. *Food Chemistry*, 123, 1090-1097
- 487 Feng, X., Li, C., Ullah, N., Hackman, R. M., Chen, L., & Zhou, G. (2015). Potential biomarker of
488 myofibrillar protein oxidation in raw and cooked ham: 3-nitrotyrosine formed by nitrosation.
489 *Journal of Agricultural and Food Chemistry*, 63, 10957-10964
- 490 Feng, X., Li, C., Jia, X., Guo, Y., Lei, N., Hackman, R. M., Chen, L., & Zhou, G. (2016). Influence
491 of sodium nitrite on protein oxidation and nitrosation of sausages subjected to processing and
492 storage. *Meat Science*, 116, 260-267
- 493 Freybler, L. A., Gray, J. I., Asghar, A., Booren, A. M., Pearson, A. M., & Buckley, D.J. (1993).
494 Nitrite stabilization of lipids in cured pork. *Meat Science*, 33, 85-96
- 495 Frida Food Data (<http://frida.fooddata.dk>), version 3, 2016, National Food Institute, Technical
496 University of Denmark. Consulted 14th of December 2016 and 6th of September 2017
- 497 Gil-Agustí, M., Esteve-Romero, J., & Carda-Broch, S. (2008). Anserine and carnosine
498 determination in meat samples by pure micellar liquid chromatography. *Journal of*
499 *Chromatography A*, 1189, 444–450
- 500 Gringer, N., Osman, A., Nielsen, H. H., Undeland, I., & Baron C. P. (2014). Chemical
501 characterization, antioxidant and enzymatic activity of brines from Scandinavian marinated herring
502 products. *Journal of Food Processing and Technology*, 5, 346
- 503 Hermann, S. S., Duedahl-Olesen, L., & Granby, K. (2015). Occurrence of volatile and non-volatile
504 N-nitrosamines in processed meat products and the role of heat treatment. *Food Control*, 48, 163-
505 169

- 506 Hermund, D. B., Yeşiltaş, B., Honold, P., Jónsdóttir, R., Kristinsson, H. G., & Jacobsen, C. (2015).
 507 Characterisation and antioxidant evaluation of Icelandic *F. vesiculosus* extracts *in vitro* and in fish-
 508 oil-enriched milk and mayonnaise. *Journal of Functional Foods*, 19, 828-841
- 509 Honikel, K.-O. (2008). The use and control of nitrate and nitrite for processing of meat products.
 510 *Meat Science*, 78, 68-76
- 511 Kanner, J. (1979). S-nitrosocysteine (RSNO), an effective antioxidant in cured meat. *Journal of the*
 512 *American Oil Chemists Society*, 56, 74-76
- 513 Lee, S. K., Mei, L., & Decker, E. A. (1997). Influence of sodium chloride on antioxidant enzyme
 514 activity and lipid oxidation in frozen ground pork. *Meat Science*, 46, 349-355
- 515 Mac Donald, B., Gray, J. I., & Gibbins, L. N. (1980). Role of nitrite in cured meat flavor:
 516 Antioxidant role of nitrite. *Journal of Food Science*, 45, 893-897
- 517 Mac Donald, B., Gray, J. I., Kakuda, Y., & Lee, L. (1980). Role of nitrite in cured meat flavor:
 518 Chemical analysis. *Journal of Food Science*, 45, 889-892
- 519 Marcuse, R. (1962). The effect of some amino acids on the oxidation of linoleic acid and its methyl
 520 ester. *Journal of the American Oil Chemists Society*, 39, 97-103
- 521 Meucci, E., & Mele, M. C. (1997). Amino acids and plasma antioxidant capacity. *Amino Acids*, 12,
 522 373-377
- 523 Milkowski, A., Garg, H. K., Coughlin, J. R., & Bryan, N. S. (2010). Nutritional epidemiology in the
 524 context of nitric oxide biology: A risk–benefit evaluation for dietary nitrite and nitrate. *Nitric Oxide*,
 525 22, 110–119

- 526 Mora, L., Escudero, E., Fraser, P. D., Aristoy, M-C., & Toldrá, F. (2014). Proteomic identification
527 of antioxidant peptides from 400 to 2500 Da generated in Spanish dry-cured ham contained in a
528 size-exclusion chromatography fraction. *Food Research International*, 56, 68–76
- 529 Morrissey, P.A., & Tichivangana, J. Z. (1985). The antioxidant activities of nitrite and
530 nitrosylmyoglobin in cooked meats. *Meat Science*, 14, 175-190
- 531 Nimalaratne, C., Lopes-Lutz, D., Schieber, A., & Wu, J. (2011). Free aromatic amino acids in egg
532 yolk show antioxidant properties. *Food Chemistry*, 129, 155–161
- 533 Oyaizu M (1986). Studies on products of browning reaction. Antioxidative activities of products of
534 browning reaction prepared from glucosamine. *Japanese Journal of Nutrition and Dietetics*, 44,
535 307- 315
- 536 Saiga, A., Tanabe, S., & Nishimura, T. (2003). Antioxidant activity of peptides obtained from
537 porcine myofibrillar proteins by protease treatment. *Journal of Agricultural and Food Chemistry*,
538 51, 3661-3667
- 539 Sebranek, J. G. (2009). Basic curing ingredients. In R. Tarte, (Ed.), *Ingredients in meat products*
540 (pp 1-24). New York: Springer Science + Business media LLC
- 541 Skibsted, L. H. (2011). Nitric oxide and quality and safety of muscle based foods. *Nitric oxide*, 24,
542 176-183
- 543 Sun, W., Zhao, H., Zhao, Q., Zhao, M., Yang, B., Wu, N., & Qian, Y. (2009). Structural
544 characteristics of peptides extracted from Cantonese sausage during drying and their antioxidant
545 activities. *Innovative Food Science and Emerging Technologies*, 10, 558–563

- 546 Tricker, A. R., & Kubacki, S. J. (1992). Review of occurrence and formation of non-volatile N-
547 nitroso compounds in food. *Food Additives and Contaminants*, 9, 39-69
- 548 Villaverde, A., Parra, V., & Estévez, M. (2014). Oxidative and nitrosative stress induced in
549 myofibrillar proteins by a hydroxyl-radical-generating system: Impact of nitrite and ascorbate.
550 *Journal of Agricultural and Food Chemistry*, 62, 2158-2164
- 551 Vossen, E., & De Smet, S. (2015). Protein oxidation and protein nitration influenced by sodium
552 nitrite in two different meat model systems. *Journal of Agricultural and Food Chemistry*, 63, 2550-
553 2556
- 554 Wang, F. S. (2003). Effect of antimicrobial proteins from porcine leukocytes on *Staphylococcus*
555 *aureus* and *Escherichia coli* in comminuted meats. *Meat Science*, 65, 615-621
- 556 Wilkinson, B.H.P., Lee, E., Purchas, R.W., & Morel, P.C.H. (2014). The retention and recovery of
557 amino acids from pork longissimus muscle following cooking to either 60 °C or 75 °C. *Meat*
558 *Science*, 96, 361-365
- 559 Xing, L.-J., Hu, Y.-Y., Hu, H.-Y., Ge, Q.-F., Zhou, G.-H., & Zhang, W.-G. (2016). Purification and
560 identification of antioxidative peptides from dry-cured Xuanwei ham. *Food Chemistry*, 194, 951-
561 958
- 562

Tables

Table 1. Initial linear antioxidant activity increase expressed in activity % (iron chelating and ABTS radical scavenging) or OD₇₀₀ (reducing power) per mg of ham or positive control (mean \pm standard deviation, n=3)

	Iron chelating	Reducing Power	ABTS radical scavenging
Sandwich ham (SH)	54 \pm 4	0.307 \pm 0.013	79 \pm 1
Dinner ham (DH)	Activity below 0	0.154 \pm 0.0002	58 \pm 1
Smoked pork saddle (SPS)	24 \pm 1	0.353 \pm 0.05	106 \pm 2
Reference ham	577 \pm 188	0.093 \pm 0.002	42 \pm 2
Positive control	24222 \pm 5332 ^a	330.57 \pm 2.33 ^b	23956 \pm 936 ^c

^a0.5mM EDTA, ^b0.5mM ascorbic acid and ^c2.5mM trolox

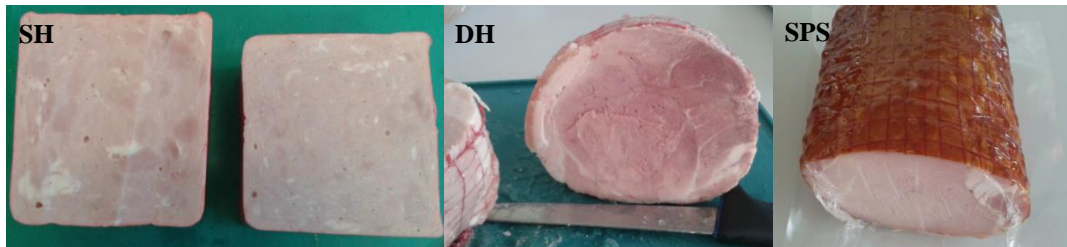
Table 2. Content of residual nitrite and ascorbic acid in the three tested nitrite-cured hams and the reference ham (mean \pm standard deviation, n=2)

	Residual nitrite (ppm)	Residual ascorbic acid (ppm)
Sandwich ham (SH)	7.3 \pm 0.1 ^a	354.0 \pm 9.5 ^{a‡}
Dinner ham (DH)	4.0 \pm 0.03 ^b	100.9 \pm 8.2 ^{b‡}
Smoked pork saddle (SPS)	10.7 \pm 0.3 ^c	445.6 \pm 18.7 ^{c‡}
Reference ham	0.3 \pm 0.03 ^d	6.4 \pm 0.2 ^d

* Values in the same column follow by different letters are significantly different (p<0.05).

[‡]n=6

Figures



579

580 Figure 1. The three nitrite-cured pork products investigated. SH: cross-section of the restructured
 581 boiled sandwich ham, DH: cross-section of boiled dinner ham and, SPS: surface and cross-section
 582 of boiled and smoked pork saddle.

583

584

585

586

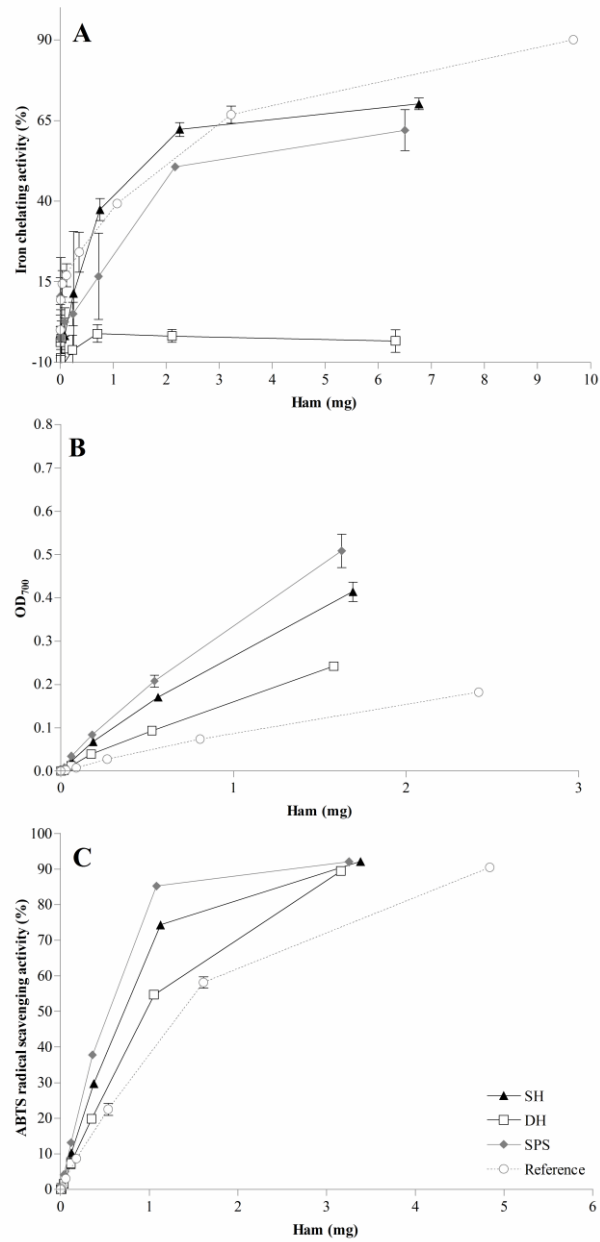
587

588

589

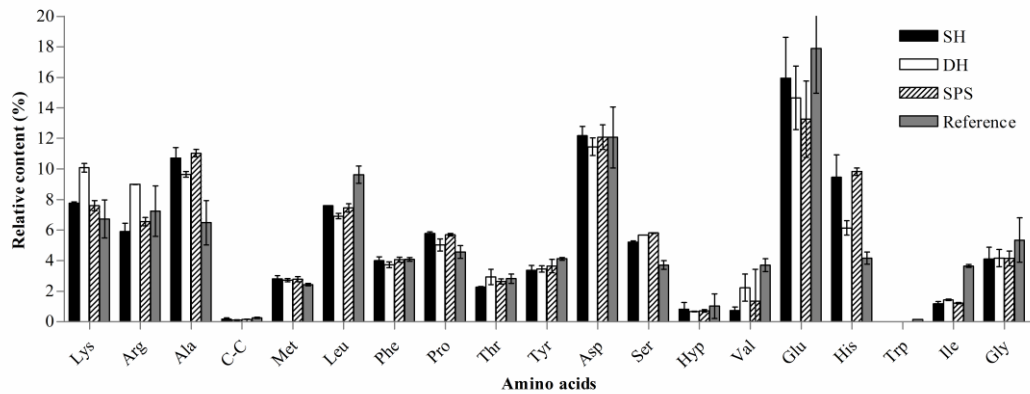
590

591



592

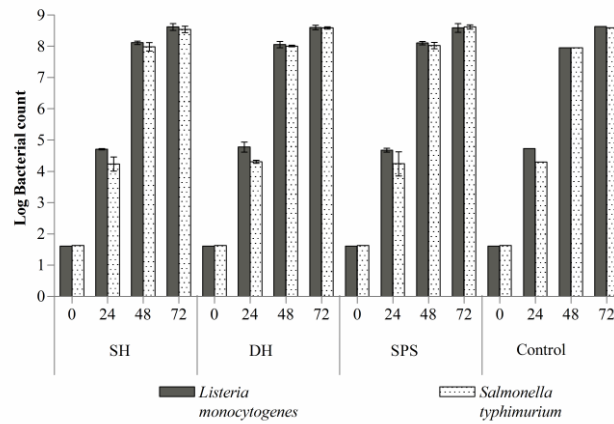
593 Figure 2. A: Iron chelating activity (%) B: reducing power activity (OD₇₀₀) and C: ABTS radical
 594 scavenging activity (%) of the ≤ 10 kDa extract from the three nitrite-cured products and the
 595 uncured reference ham. The activity of the extracts is plotted as function of mg ham used for
 596 making the analysed volume of extract.



597

598 Figure 3. The relative content of the total amino acids in % in the sandwich ham (SH), the dinner
 599 ham (DH), the smoked pork saddle (SPS) and the uncured reference ham (Reference).

600



601

602 Figure 4. Log10 transformed bacterial count from the growth inhibition study (study of
 603 antimicrobial activity of ham extract) on *Listeria monocytogenes* and *Salmonella typhimurium* after
 604 0, 24, 48 and 72 hours incubation. Control indicates ham extract being substituted for equivalent
 605 amounts FKP.

606

Paper II

Pedersen, S. T., Duedahl-Olesen, L. & Jessen, F. Characterization of low molecular weight peptidic extracts from nitrite-cured cooked pork products. *Submitted to Journal of the Science of Food and Agriculture.*

**Characterization of low molecular weight peptidic extracts
from nitrite-cured cooked pork products**

Journal:	<i>Journal of the Science of Food and Agriculture</i>
Manuscript ID	Draft
Wiley - Manuscript type:	Research Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Pedersen, Sabine; Technical University of Denmark, National Food Institute Duedahl-Olesen, Lene; Technical University of Denmark, National Food Institute Jessen, Flemming; Technical University of Denmark, National Food Institute;
Key Words:	ham, ABTS radical scavenging, reducing power, amino acids, nitrite-curing, FPLC

SCHOLARONE™
Manuscripts

Title: Characterization of low molecular weight peptidic extracts from nitrite-cured cooked pork products

Authors: Sabrine Tauber Pedersen^a, Lene Duedahl-Olesen^a & Flemming Jessen^a.

^a National Food Institute, Technical University of Denmark, Søtofts Plads Building 227, DK-2800 Kongens Lyngby, Denmark

Running title: Characterization of origin of antioxidant activity in extracts from cured hams

***Corresponding author:**

Flemming Jessen

Postal address:

National Food Institute,
Technical University of Denmark
Søltofts Plads Building 221, ground floor, room 050
DK-2800 Kgs. Lyngby

E-mail:

fjes@food.dtu.dk

Declaration of conflict of interest: none

Abstract

BACKGROUND: Nitrite-cured cooked pork products constitute an immense variety of products but a common characteristic is a long shelf-life attributed to by the curing process. In order to contribute to a better understanding of the antioxidative mechanisms occurring in nitrite-cured cooked pork products to ultimately optimize nitrite addition the aim of this study was to investigate the contribution to antioxidant activity of other compounds than residual ascorbate and nitrite from aqueous ≤ 10 kDa extracts of cold-stored nitrite-cured cooked pork products.

RESULTS: By means of size exclusion chromatography (SEC), *in vitro* antioxidant assays and constituent amino acids analysis it was demonstrated that the extracts exhibited antioxidant activity in the form of reducing power and ABTS radical scavenging activity (with different kinetics). It was also demonstrated that this activity was concentrated to only a few fractions from the SEC separation. The SEC results indicated the antioxidant activity to originate from compounds < 6.5 kDa and could include small peptides and free amino acids e.g. the aromatic phenylalanine and tyrosine. Furthermore, the extracts were high in the antioxidant amino acids histidine and methionine.

CONCLUSION: The antioxidant activity did neither stem from residual nitrite nor ascorbate but it could be associated with nitrite-related compounds formed during curing and heating. This shows that we need to expand our perception of the cause of oxidative stability in nitrite-cured cooked hams to include more than just nitrite and ascorbate.

Keywords: ham, ABTS radical scavenging, reducing power, amino acids, nitrite-curing, FPLC

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Introduction

Curing of meat is a well-established procedure for adding flavor and prolonging shelf-life of meat products. In its general form meat curing consists of adding sodium chloride and salts of nitrite (or nitrate) and ascorbate to raw meat, however, many curing processes also involves addition of spices and further downstream processing as cooking and smoking. The chemistry of nitrite and ascorbate in processed meat is complex and at times not fully understood. However, ascorbate as an electron donor, is an effective antioxidant that through its relatively unreactive oxidized form (dehydroascorbic acid) can terminate propagation of free radical reactions¹. Chloride is in many biological systems considered an unspecific prooxidant and although nitrite, as a natural electron acceptor, can be considered a strong prooxidant several mechanisms have been suggested in favor of nitrite as an active antioxidant². However, it is well established that their combined addition inhibits lipid oxidation².

In a recent study aqueous low molecular weight extracts ($\leq 10\text{kDa}$) of nitrite-cured cooked hams were demonstrated to exhibit *in vitro* antioxidant activity³. The origin of the antioxidant activity was not fully elucidated and could as such originate from residues of the added antioxidants. Activity originating directly from residual nitrite was rejected as the antioxidant activity of nitrite primarily is exerted through other chemical forms e.g. NO. However, besides residual ascorbate the results also indicated that compounds, potentially related to nitrite, formed during processing (cooking and curing) could have contributed to the detected activity. Furthermore, certain peptides or free amino acids may also have added to the detected antioxidant activity³.

Many peptides of 2-20 amino acids in length have been found to exhibit a range of bioactivities including antioxidant activity⁴. Several amino acids such as histidine, methionine, tryptophan, tyrosine and cysteine have generally been accepted as possessing antioxidant properties, a capacity demonstrated in free form and/or enclosed in peptides⁵⁻⁷. Small antioxidant peptides have

previously been identified in animal based foods. The most frequently mentioned are the antioxidative endogenous histidyl dipeptides, carnosine and anserine which have been identified in many different meats including pork meat⁸. However, other small peptides displaying antioxidant properties have also been found in fresh pork ham⁹, porcine myofibrillar proteins⁷, sausages¹⁰ and cured hams^{11,12}. Most attempts to characterize and identify antioxidant peptides has been in raw sausages or hams subjected to a drying or ripening step characterized by extensive protein hydrolysis by endogenous enzymes or microorganisms or alternatively after enzymatic treatment on the final product (or extracts hereof) – all resulting in the presence of immense amounts of small peptides (and free amino acids). In cooked nitrite-cured hams this profound protein breakdown do not occur. Thus, in order to contribute to a better understanding of the antioxidative mechanism occurring in nitrite-cured cooked pork products, the aim of this study was to investigate the potential origin of antioxidant activity from other compounds than residual ascorbate and nitrite. It was hypothesized that aqueous ≤ 10 kDa extracts of nitrite-cured cooked hams contains compounds of peptide origin contributing to antioxidant activity.

Materials and method

Materials

For nitrite determination and antioxidant assays sodium nitrite, ascorbic acid, and 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), were acquired from Sigma-Aldrich (St. Louis, MO, USA). Ethylenediaminetetraacetic acid disodium dihydrate (EDTA) were from Merck Millipore (Billerica, MA, USA) and (\pm)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) from Fluka Chemika (Deisenhofen, Germany). Cytochrome C from equine heart (CytC), aprotinin from bovine lung, triglycine (Gly₃), L-tryptophan (Trp) and L-histidine (His), L-methionine (Met), L-tyrosine (Tyr), L-proline (Pro) and L-glutamic acid (Glu) were from Sigma-Aldrich while L-cysteine (Cys) and L-phenylalanine (Phe) were from Merck Millipore and

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

glycine (Gly) were supplied by AppliChem GmbH (Darmstadt, Germany). L-alanine (Ala) was from Fluka Chemika. Remaining chemicals and reagents employed were of analytical grade and acquired from VWR International (Radnor, PA, USA), Merck Millipore, Sigma-Aldrich or Fluka Chemika.

Hams

The sample material comprised three different types of commercially available nitrite-cured cooked pork products: a restructured sandwich ham (SH), a whole-muscle boiled dinner ham (DH) and a whole-muscle smoked pork saddle (SPS). SH was added 60 ppm nitrite while DH and SPS were added 120ppm nitrite. At the laboratory, four days after production, the pork products were cut into smaller pieces, vacuum-packed, stored at 5°C (from now on referred to as day 0) and samples were periodically taken and moved to -80°C until further analysis.

The samples were tested without any further heat treatment. Any remaining skin and excess subcutaneous fat on the boiled dinner ham were trimmed before mincing. The smoke-colored surface of the pork saddle was not removed as it is expected to be eaten by the majority of Scandinavian consumers, unlike the fat and skin on the dinner ham.

Extraction of water soluble ham components and preliminary fractionation

Water soluble compounds were extracted from the cured hams by homogenising 79.4-114.3g of minced ham in 400mL of deionized water, dH₂O, for 1min with an Ultra Turrax (T 25 Basic ULTRA-TURRAX® mounted with S25N -18G dispersing element, IKA®-WERKE GmbH &Co. KG, Staufen, Germany) at 13,500rpm and subsequently adding up to a total volume of 1.5L. An aqueous ≤10kDa extract was collected by submerging five times 10cm dialysis membrane tubes (Spectra/Por 6, MWCO 10,000Da, Spectrum Lab. Inc, CA, USA) containing 16.5mL dH₂O (corresponding to 50% membrane volume) into the meat/water homogenate. After 24h of magnetic stirring at 5°C, the dialysate (extract) in the 5 membranes were pooled and stored at -80°C until

further analysis. Later the protein content was determined using the PierceTM Modified Lowry Protein Assay Kit in microplate format according to manufactures description (Thermo Scientific, PierceTM) using Bovine Serum Albumin (BSA) (Albumin Standard Ampules, 2 mg/ml) supplied with the kit as an external standard.

Size exclusion chromatography (SEC)

Size exclusion chromatography (SEC) was performed on the aqueous ≤ 10 kDa extract on fast performance liquid chromatography (FPLC) equipment (Äkta Purifier system with Frac 950 collector, GE Healthcare Life Sciences, Little Chalfont, UK). In short, 100 μ L or 200 μ L of the extract were injected three times onto a SuperdexTM peptide 10/300 GL column (GE Healthcare), using a 100 mM ammonium acetate, pH 8 as running buffer at a flow rate of 0.5 mL/min. Eluting compounds were detected at 215 nm (peptide bonds), 254 nm (phenylalanine) and 280 nm (aromatic ring e.g. tyrosine, tryptophan, phenylalanine). CytC (12.3 kDa), Aprotinin (6.5 kDa), Gly₃ (189 Da) and the amino acids glycine (75 Da), cysteine (121 Da), histidine (155 Da), phenylalanine (165 Da) and tryptophan (204 Da) were used as external standards. Elution volumes and molecular weights of standards are not fully related as the column possesses some hydrophobic affinity properties apart from being a size exclusion column. For each extract-run, 94 fractions (SEC fractions) of 320 μ L each were collected into a 96 well microplate and the plate was stored at 5°C until the run for the third injection, of the same extract, had finish. The content of each well were then pooled, in Eppendorf tubes, with the corresponding well in the other two plates of the same sample and stored at -20°C until further analysis.

Chemical analyses

Nitrite

The day before analysis, approximate 100 g of ham sample stored at -80°C was thawed, minced two times 5 sec, and re-vacuum packed before being returned to -80°C. Extraction and measurement of

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

nitrite in the cured cooked pork products was done according to Herrmann, Duedahl-Olesen & Granby (2015)¹³. The samples were analyzed in a random order in duplicate. External calibration was performed in the range 0.25-100µg/mL NaNO₂. As the nature of the added nitrite was unknown in SH all results are presented as ppm nitrite-ion by the relationship 1 g nitrite ion = 1.50g NaNO₂.

Salt

The salt content of the pork extracts was determined through potentiometric titration of chlorine ions/chloride using AgNO₃ according to AOAC Official Method 976.18 (Salt (Chlorine as Sodium Chloride) in Seafood. Potentiometric method, 2000).

Protein

The protein content of the collected SEC separated fractions were determined using the PierceTM BCA Protein Assay Kit according to manufactures description for determination in microplates (Thermo Scientific, PierceTM) using Bovine Serum Albumin (BSA) (Albumin Standard Ampules, 2 mg/ml) supplied with the kit as an external standard.

Amino acid profile (total and free)

The total amino acid content was analyzed by subjecting 0.5mL of the aqueous ≤10kDa extract to microwave-assisted acidic hydrolysis (6M HCl) for 1h at 110°C and 500W followed by 10min of cooling. The samples were diluted with 1M NaCO₃ and filtered through a 0.2µm syringe filter (Q-max PTFE, Ø13mm, Frisenette ApS, Knebel, Denmark) before derivatization using the EZ:FaastTM Amino Acid Analysis kit from Phenomenex® (Torrance, CA, USA). The derivatized samples were analyzed by LC-(APCI)-MS (Agilent 1100, Agilent Technology) according to the procedure described by Farvin, Baron, Nielsen, Otte & Jacobsen (2010)¹⁴.

Free amino acid content was determined by introducing the extracts at the derivation step and otherwise proceeding as described above. The analysis was done in duplicate.

161

162 *Antioxidant assays*

163 Potential antioxidant properties of the SEC fractions were analyzed using two *in vitro* assays for
 164 antioxidant activities; reducing power and ABTS-radical scavenging. The assays were conducted in
 165 triplicate and all measurements were performed using a Synergy 2 Multi-Mode Microplate Reader
 166 (BioTek® Instruments, Inc., Winooski, VT, USA). Before analysis the SEC fractions were thawed
 167 at 5°C, overnight.

168 The reducing power was determined according to the procedure described by Oyaizu (1986)¹⁵ with
 169 some modifications and adjusted to microplate detection. First, equal parts of each SEC fraction,
 170 0.2M phosphate buffer (pH 6.6) and 1% potassium ferricyanide were mixed and incubated for
 171 20min at 50°C. Subsequently, 1 part of 10% TCA was added. Then, 100µL of the sample mixture
 172 was mixed with 100µL dH₂O and 20µL of 0.1% ferric chloride, and incubated 10min at room
 173 temperature. The absorbance was measured at 700nm. Ascorbic acid (0.5mM) was used as positive
 174 control and dH₂O and 100mM ammonium acetate were used as blanks. Results are expressed as

$$OD_{700} = Abs_{700nm}(Sample) - Abs_{700nm}(blank)$$

175 The radical scavenging activity was determined according to the method described by Gringer,
 176 Osman, Nielsen, Undeland & Baron (2014)¹⁶. Trolox (2.5mM, in 96% ethanol) was used as a
 177 positive control. Ethanol (96%) and 100mM ammonium acetate were included as blanks for the
 178 positive control and sample, respectively. The ABTS radical scavenging activity (%) is expressed as
 179 the relative decrease in color/absorbance (734nm):

$$ABTS \text{ radical scavenging activity } (\%) = \frac{Abs_{blank} - Abs_{sample}}{Abs_{blank}} \cdot 100\%$$

180 *Statistical analysis*

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Measurements were carried out in triplicate (unless it is stated otherwise). Results are presented as mean \pm standard deviation. All statistical analyses were conducted using the GraphPad Prism® software Ver. 4.03 or R version 3.3.0 with $P < 0.05$. Results were compared using one-way ANOVA with Tukeys posttest.

Results and discussion

Nitrite was measured at several time points during storage of the cured hams to identify the time point at which residual nitrite reached a low level to limit any potential interference e.g. ensuring that formation of nitrite related reaction products will have reached its maximum/steady level.

The residual nitrite concentration in the tested hams decreased over time, as was expected, and reached a common low level after 37 days (Figure 1). The smoked pork saddle (SPS) had the highest concentration of residual nitrite at 10.7 ± 0.3 ppm at day 0 followed by the sandwich ham (SH) and boiled dinner ham (DH) at 7.3 ± 0.1 ppm and 4.0 ± 0.03 ppm, respectively. Cassens (1997)¹⁷ reported residual nitrite levels of 7 ± 1 ppm and 4 ± 1 ppm in commercial sliced hams which was similar to SH and DH. After end processing and heat treatment residual nitrite has been reported to be reduced to $\leq 35\%$ of the originally added amounts¹⁸, fitting with the current data (day 0). The product difference in residual nitrite at day 0 did not correlate with added nitrite as DH and SPS were added twice the amount of nitrite compared to SH. After the 37 days the residual nitrite concentration reached a low level around 1-2 ppm for all hams. Pérez-Rodríguez, Bosch-Bosch and García-Mata (1996)¹⁹ measured residual sodium nitrite in pork frankfurter sausages over time and found residual sodium nitrite in a sausage (125 ppm NaNO₂, 500 ppm Na-ascorbate, 2.2% NaCl) to be fairly constant just below 7 ppm NaNO₂ (equivalent < 4.7 ppm NO₂-ion) after 27 days of storage at 3°C (NaNO₂ content was extrapolated from Fig. 2 in Pérez-Rodríguez *et al.* (1996)¹⁹). Also in the

lean as well as fat part of bacon (156ppm NaNO₂, 500ppm ascorbate, 2.5% NaCl) residual sodium nitrite were close to zero after 35 days at 2°C²⁰.

From the hams stored at 5°C for 37 days, ≤10kDa aqueous extracts were produced and used for characterization. The ≤10kDa extracts were produced from meat/water homogenates made from slightly different amounts of meat. Yet, the protein content were very similar between the extracts, (SPS: 0.17mg/mL, DH: 0.17mg/mL, SH: 0.16mg/mL) and did not correlate with the difference in amount of meat used. Based on this the extracts were regarded to be comparable. However, due to much lower absorbance signals from the ≤10kDa extract from DH compared to SH and SPS (data not shown) when injecting 100μL into the FPLC it was decided to inject twice the volume of DH making the signal magnitude of the absorbance profile for DH comparable to SH and SPS. The extracts all had similar SEC profiles (Figure 2) according to the absorbance at 215nm, and were characterized by four main peaks (I: 18.5-19.9mL, II: 19.9-21.4mL, III: 21.4-22.9mL and IV: 25.3-27.2mL), a shoulder eluting before peak I and a peak eluting after peak III only base-line separated in SH (Figure 2 top panel).

Only minor signals were detected from aromatic rings absorbing at 280nm and these correlated nicely with peak II-IV. The strongest signal at 280nm was achieved in peak III in DH, Figure 2 middle panel. Aromatic rings were also detected at 254 nm (optimum phenylalanine). This signal generally correlated well with the elution pattern from the 280nm signal yet gave rise to much higher maximum absorbance in the range 15-500mAU compared to 2-90mAU at 280nm.

Although the SEC chromatograms show similar profiles between the three samples (non-quantitatively) the mutual relationship between the peak maximum differed between the hams (Figure 2). As both DH and SPS are whole muscle products and were both added the same amount of nitrite - 120ppm (information given by manufacture) corresponding to twice the amount added to SH – they would be expected to be the most similar. However, if focusing on 254nm and 280nm

curves the inter-peak relationship shows greater resemblance between SPS and SH. This is also contradictory to the fact that the smoking of the SPS could potentially cause formation of aromatic smoke components e.g. phenolic compounds and polycyclic aromatic hydrocarbons (PAH)^{21,22}, possibly increasing the aromatic absorbance signal compared to non-smoked hams. For example Chen, Wang and Chiu (1996)²³ detected 16 different PAHs in several meat products, including smoked pork, at 254nm after separation on a C18 column.

CytC and aprotonin eluted before the sample constituents indicating the aqueous ham extracts only contained compounds, detectable by the current FPLC method, that were smaller than 6.5kDa (Figure 2). Thus, the presence of peptides with antioxidant properties in the extracts is not unlikely as antioxidant peptides seldom contain more than 20 amino acids per molecule and have masses below 6000Da^{4,24}. For example Escudero *et al.*, (2012)¹¹ found a peptide fraction <1700Da from dry cured ham to possess antioxidant activity while Li, Han & Chen (2008)²⁵ indicated that peptides of 500-1500Da exhibited stronger antioxidant activity than both smaller and larger peptides. Peak I including the anterior shoulder, present in all three extracts, spanned the elution of Gly₃ (189Da → 18.4mL), glycine (75Da → 19.3mL), and towards the end cysteine (121Da → 19.7mL) and histidine (155Da → 19.9mL). The elution of the remaining two standards, phenylalanine (165Da) and tryptophan (204Da), which both contain an aromatic ring, correlated with the sample elution of peak II (280 and 254nm) and a minute 280nm-peak eluting at approximately 28.5mL (Figure 2).

The inconsistency in relation between the sizes of standards and their elution volumes is due to column properties (see Materials and Methods)

The results from *in vitro* determination of reducing power and ABTS radical scavenging activity in the collected SEC separated fractions are presented in Figure 3. Reducing power (Figure 3A) is for all three extracts concentrated around two fraction-peaks, P1 and P2. DH showed the highest reducing power activity of the three, in fraction-peak P2, and SPS the lowest. The higher signal

from DH might have been due to the injection of 200 μ L of this extract to the FPLC which as such created a different foundation for the collected SEC fractions compared to the remaining two where only 100 μ L were injected. It is noteworthy that P1 fell within the range of peak I (including the anterior shoulder) in the SEC chromatogram (Figure 2). Yet more notable that reducing power fraction-peak, P2, perfectly aligned with chromatographic peak, II, with a strong signal at all three wavelengths, but in particular 215nm and 254nm (Figure 2). This indicated the presence of peptide bonds and aromatic rings among the eluting compounds in the antioxidant active (reducing power) fraction-peak. Furthermore, P2 and peak II also coincided with the elution of the external phenylalanine standard (Figure 2 top panel). Thus, it can be speculated that the reducing power noted in the tested ham extracts may in full (or partly) have been caused by phenylalanine or alternatively small peptides. As a result of the ring structure the aromatic amino acids phenylalanine, tyrosine and tryptophan can act as proton donors for free radicals deficient in electrons and efficiently scavenge them²⁶. Broncano *et al.* (2012)¹⁰ for example suggested that a tripeptide Phe-Gly-Gly with the N-terminal phenylalanine is capable of scavenging DPPH radicals. Despite studies showing that antioxidants may display different efficiency in scavenging the ABTS and the DPPH radical²⁷ it would be expected, based on the above, that peak II would correlate with a fraction-peak in the ABTS radical scavenging profile rather than with a reducing power fraction-peak. Thus, if the assumption that phenylalanine is contributing to the discovered reducing power activity is valid, then the aromatic amino acids including phenylalanine may potentially also be able to contribute to the reduction of iron. However, no reducing power activity could be detected for free phenylalanine at concentrations ≤ 1 mg/mL while tryptophan exhibited some activity (OD₇₀₀=0.06 at 1mg/mL) (supplementary data, Figure 1S).

Incubation time used for ABTS radical scavenging activity assays varies a lot between laboratories and also the procedure differs slightly. Some studies apply 1-10min^{16,28,29} while others report up to

1h incubation³⁰. Thus ABTS radical scavenging activity was measured after 1min (Figure 3B) incubation to detect a fast response and 31min (Figure 3C) incubation to look for any slower acting activities. After 1min incubation activity was detected in fraction-peak, P3, at 21.1-23.1mL. After additional 30min incubation the ABTS radical scavenging activity in P3 had only increased slightly from 17.9 to 23.1% for SPS and 20.6 to 28.2 % for DH while it increased a bit more for SH from 9.2 to 24.6%. However, after the additional 30min incubation ABTS radical scavenging activity also appeared in the fraction-peak P4. This clearly showed different reaction kinetics of the mixture of active compounds enclosed in P3 and P4. Thus, it should be considered for future studies to measure ABTS radical scavenging activity at more time points to gain more information on antioxidant compound kinetics. Overall DH displayed the strongest ABTS radical scavenging activity however as argued before this could be due to the larger injection volume. Aside from this SH showed higher ABTS radical scavenging activity than SPS in P4 while the relation was reversed in P3.

The position of ABTS radical scavenging activity fraction-peak P4 corresponded to reducing power fraction-peak P1 (Figure 3A and C) and thus, also aligned with the large peak I (Figure 2), in the SEC chromatogram yet slightly shifted to the right. At this point of elution the absorbance at 254nm and 280nm was very limited and thus led to idea that the antioxidant activity in peak I/P1 could involve the action of small peptides but also free amino acids (other than the aromatic). The longer incubation time required for P4 was also evident especially for the free amino acids histidine, proline and glycine when analyzed in concentration >0.02mg/mL but not at concentration equivalent to the content of the individual free amino acids in the extracts (Figure 4 and supplementary data, Figure 2S).

The ABTS radical scavenging activity detected after 1min incubation, P3, (Figure 3) aligned nicely with peak III absorbing at both 215nm and 254nm/280nm (Figure 2). Therefore some of the

detected activity may again have been the action of a peptide or a compound containing some sort of aromatic ring. This could have been from the amino acids tyrosine as the elution of the other aromatic amino acids phenylalanine and tryptophan was already established. Yet nothing conclusive can be said on this matter as solubilization and therefore injection of tyrosine was unsuccessful. However, solutions of tyrosine (although turbid) were found to be highly potent at color reduction even at low concentrations (15-22% ABTS radical scavenging activity at 0.002mg/mL, see supplementary data, Figure 2S).

In spite of the SEC separation of the extracts resulted in four main absorbance peaks, the antioxidant activity was limited to three of these as described above. It is of no surprise that the extract would contain compounds of no antioxidant activity. Yet it is relatively unexpected that compounds giving rise to an absorbance peak at 254nm and 280nm (peak IV in Figure 2) would not possess some antioxidant activity as the most common antioxidants from food matrixes contain aromatic rings. However, it cannot be ruled out that a potential antioxidant activity from compounds in this peak simply is not detectable by the two *in vitro* antioxidant assays applied.

The importance of nitrite for the oxidative stability of meat inspires the hypothesis that hams added more nitrite would exhibit more antioxidant activity. However, comparing SH and SPS (same volume used for SEC fractionation) this was not unambiguous substantiated. In the peak-fractions P2 (reducing power) and P3 (ABTS radical scavenging 1min peak) the hypothesis of more added nitrite being equivalent to more activity was supported. On contrary P4 (ABTS radical scavenging 31min peak) in SH showed stronger activity than SPS. Morrissey & Tichivangana (1985)³¹ also demonstrated a significant reduction in lipid oxidation/TBA values at the addition of 20ppm nitrite to pork (and other meat types) which further decreased to a 12-fold reduction at the addition of 200ppm. On contrary MacDonald, Gray & Gibbins (1980)³² found higher concentrations of nitrite to cause more pronounced prooxidant effect on lipid oxidation, but the level of nitrite in commercial

meat products was too low to exert this effect. Thus, the current data does not rule out the importance of nitrite in regards to presence/creation of compounds possessing antioxidant activity yet neither does it confirm its insignificance.

Ascorbic acid/ascorbate is a known antioxidant³³, which was also added to all three hams, and as such could have disturbed the measurements. Extracts, similar to the current ham extracts, however produced from sausages of varying nitrite and ascorbate content were fractionated by SEC (see supplementary data, Figure 3S). A massive peak protruded at 18.5-20.2 mL, which coincided with peak I observed here, which did not materialized when ascorbic acid was not added and only resulted in a minor peak when added in smaller amount (240ppm). However, as would be expected from the ring structure of ascorbic acid the peak was due to high absorbance at 254 and 280nm. As no such peak were evident in the current study it is considered unlikely that the detected reducing power and ABTS radical scavenging is caused by residual amounts of the ascorbic acid added to the hams.

The total amino acid profile was relatively similar between the three extracts and for 13 of the 19 investigated amino acids no statistical differences were detected (Figure 4A). Comparison of the distribution of the individual amino acids (percent of total amino acid) in the extract to the distribution found in raw and cooked (60 and 75°C) pork muscle³⁴ and ham products in the Danish food composition database (Frida Food Data - <http://frida.fooddata.dk/>³⁵) revealed some striking differences. The profiles from the literature were dominated by glutamic acid (glutamic acid + glutamine) and aspartic acid (aspartic acid + asparagine) a result also found during previous investigations of the three ham products used in the current study³. However, the extracts were dominated by histidine, alanine, methionine, glutamic acid and glycine in decreasing order. Both histidine and methionine are among the amino acids frequently mentioned in regard to peptide related antioxidant activity and thereby supported the expectation of finding higher antioxidant

activity in the ≤ 10 kDa fraction compared to the entire ham. It is also noteworthy that carnosine (226.24 Da), a dipeptide found in meat possessing antioxidant activity, is made up of exactly alanine and histidine.

The importance of aromatic amino acids in antioxidative peptides has been emphasized previously yet the presence of other amino acids in the sequence e.g. glycine, cysteine and the acidic amino acids may also be of great importance for antioxidant properties in a peptide²⁶. However, from an alignment of the sample and standard SEC chromatograms and the antioxidant activity profiles no information can be drawn concerning small antioxidative peptides of this particular composition. Although SH and SPS contains significantly more histidine and alanine no direct correlation can be made from the content of the amino acids and the detected antioxidant activity due to the difference in starting material for the assays.

Several amino acids have also been found to possess antioxidant properties as free amino acids³⁶ including histidine, tryptophan and cysteine^{5,6}. The latter two showed as high ABTS radical scavenging activity at 0.05 mg/mL as the positive control (2.5 mM Trolox) while cysteine also had the strongest reducing power of the ten tested amino acids (supplementary data, Figure 2S). The content of free amino acids was, as expected, very low: 0.0788, 0.0799 and 0.0684 mg amino acid/g extract in SH, SPS and DH respectively. However, the amount of free amino acids accounted for 23–27% of the total amino acid composition of the extract. The free amino acid pool was more even distributed between the individual amino acids compared to the total amino acids (Figure 4B).

Overall the free amino acids profiles were very similar but for seven amino acids the content was significantly different in one ham extract compared to the remaining two. SPS contained higher amounts of lysine, tyrosine and aspartic acid compared to SH and DH. DH contained lower amounts of hydroxyproline, methionine and threonine, compared to in the remaining extracts while SH contained lower amount of valine. Although alanine generally was dominating the free amino

acid profile, alanine in the free form is seldom mentioned in regard to antioxidant activity but mainly in connection with carnosine. Also, alanine did not demonstrate any reducing power or ABTS radical scavenging activity in concentration $\leq 1\text{mg/mL}$ (supplementary data, Figure 2S). However, alanine was by Karel *et al.* (1966)⁵ found to exhibit antioxidant activity. Thus, it cannot be ruled out that alanine contributed to the peptide related antioxidant pool yet the extent of its contribution is unclear. Consequently, it must be assumed that alanine together with the remaining free amino acids, all jointly contributed to the observed antioxidant activity.

The fractions giving rise to the main peaks in the SEC chromatograms that also showed antioxidant activity were pooled and the antioxidant activities were normalized to the protein content (Table 1). The slight shift between P1 and P4 was disregarded and the two peak-fractions were pooled as one. Based on protein content the rank order between the samples in P1 changed from DH showing a slightly higher reducing power than SH with SH possessing double the protein specific reducing power of DH (Table 1). Considering the injected amount of protein (SPS: $17\mu\text{g}$, DH: $34\mu\text{g}$, SH: $16\mu\text{g}$) it appeared that the changes in the previously similar OD_{700} of SH and DH almost matched the difference in injected amount of protein. This could be an indication of the reducing power detected in P1 being caused by proteins. The same correlation to protein content was not evident for P2. However, the biuret reaction constituting the foundation for the BCA method is limited by not detecting proteins smaller than tripeptides although studies have suggested that dipeptides also affect the color formation^{37,38}. Anyway, the method does not detect free amino acids making the previously presented hypothesis of the activity originating from phenylalanine in P2 still applicable. Although SPS had the highest content of free phenylalanine (Figure 4B) it was not significantly different from the two remaining ham extracts. Taking this and any uncertainties introduced with the normalization into consideration it must be noted that DH demonstrated almost twice the protein specific reducing power of SPS (double amount injected of DH). Also that the relationship between

protein specific reducing power for SPS and DH ($68/73=0.93$) matched the relationship between injected protein ($16/17=0.94$)

The protein specific ABTS radical scavenging activity in P4 was the highest in SH, very similar in DH, however, only half in SPS (Table 1). Thus, a correlation between the ABTS radical scavenging activity detected in P4 and protein in general is not obvious. Neither does anything in the free amino acid profile seem to offer a clear explanation. Even though the free histidine content is highest in SH (Figure 4B) the standard deviation on DH, and the fact that more was injected of this extract, would lead to the expectation of DH showing the highest protein specific ABTS radical scavenging activity if it was to be caused by free histidine. From the current data it is not possible to determine if the detected activity originated from proteins or free amino acids. It can be speculated that the activity in the SH and DH are related to protein content as they approach each other numerically and that the higher protein specific ABTS radical scavenging activity in SPS may be related to compounds not present in DH and SH that could contribute to ABTS radical scavenging activity. As SPS is the only smoked ham it is sensible to assume that such antioxidant compounds could originate from this particular processing step as several smoke phenols have been demonstrated to be active antioxidants²². Furthermore, it can be speculated whether nitrogen oxides from the smoke could have entered into some of the many reactions leading to the antioxidant effects of nitrite.

Conclusion

Conclusively, the characterization of the aqueous low molecular weight extracts ($\leq 10\text{kDa}$) from the three nitrite-cured cooked pork products revealed that the detected antioxidant activity likely originated from a broad variety of components. Components both natural to the meat e.g. peptides and free amino acids, and components related to the processing e.g. smoke components and nitrite-

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

related compounds formed during curing and heating. However, further investigations are needed in order to fully determine the extent of the antioxidant effect of the individual contributors - knowledge needed to better understand the antioxidative mechanism in nitrite-cured cooked pork so that nitrite addition may be optimized in the long term.

Funding

This work was supported by a grant from Norma and Frode S. Jacobsens Fond.

References

1. Bendich A, Machlin LJ, Scandurra O, et al. The antioxidant role of vitamin C. *Adv Free Radic Biol Med* 1986; 2: 419–444.
2. Skibsted LH. Nitric oxide and quality and safety of muscle based foods. *Nitric Oxide* 2011; 24: 176–183.
3. Pedersen ST, Baron CP, Gobba C De, et al. Antioxidative and antimicrobial activity of extracts from nitrite-cured cooked pork products. *Submitt to Food Chem*.
4. Korhonen H, Pihlanto A. Bioactive peptides: Production and functionality. *Int Dairy J* 2006; 16: 945–960.
5. Karel M, Tannenbaum SR, Wallace DH, et al. Autoxidation of methyl linoleate in freeze-dried model systems. III. Effects of added amino acids. *J Food Sci* 1966; 31: 892–896.
6. Marcuse R. The effect of some amino acids on the oxidation of linoleic acid and its methyl ester. *J Am Oil Chem Soc* 1962; 39: 97–103.
7. Saiga A, Tanabe S, Nishimura T. Antioxidant activity of peptides obtained from porcine myofibrillar proteins by protease treatment. *J Agric Food Chem* 2003; 51: 3661–3667.
8. Mora L, Sentandreu MÁ, Toldrá F. Contents of creatine, creatinine and carnosine in porcine muscles of different metabolic types. *Meat Sci* 2008; 79: 709–715.
9. Park SY, Chin KB. Antioxidant activities of pepsin hydrolysates of water- and salt-soluble protein extracted from pork hams. *Int J Food Sci Technol* 2011; 46: 229–235.
10. Broncano JM, Otte J, Petró MJ, et al. Isolation and identification of low molecular weight antioxidant compounds from fermented ‘chorizo’ sausages. *Meat Sci* 2012; 90: 494–501.

- 1
2
3
4 449 11. Escudero E, Aristoy M-C, Nishimura H, et al. Antihypertensive effect and antioxidant
5
6 450 activity of peptide fractions extracted from Spanish dry-cured ham. *Meat Sci* 2012; 91: 306–
7
8 451 311.
9
10
11 452 12. Mora L, Escudero E, Toldrá F. Characterization of the peptide profile in Spanish Teruel,
12
13 453 Italian Parma and Belgian dry-cured hams and its potential bioactivity. *Food Res Int* 2016;
14
15 454 89: 638–646.
16
17
18 455 13. Herrmann SS, Duedahl-Olesen L, Granby K. Occurrence of volatile and non-volatile N-
19
20 456 nitrosamines in processed meat products and the role of heat treatment. *Food Control* 2015;
21
22 457 48: 163–169.
23
24
25 458 14. Farvin KHS, Baron CP, Nielsen NS, et al. Antioxidant activity of yoghurt peptides: Part 2 –
26
27 459 Characterisation of peptide fractions. *Food Chem* 2010; 123: 1090–1097.
28
29
30
31 460 15. Oyaizu M. Studies on products of browning reaction. Antioxidative activities of products of
32
33 461 browning reaction prepared from glucosamine. *Japanese J Nutr Diet* 1986; 44: 307–315.
34
35
36 462 16. Gringer N, Osman A, Nielsen HH, et al. Chemical characterization, antioxidant and
37
38 463 enzymatic activity of brines from scandinavian marinated herring products. *J Food Process*
39
40 464 *Technol*; 5. Epub ahead of print 2014. DOI: 10.4172/2157-7110.1000346.
41
42
43 465 17. Cassens R. Residual nitrite in cured meat. *Food Technol* 1997; 51: 53–55.
44
45
46 466 18. Honikel K-O. The use and control of nitrate and nitrite for the processing of meat products.
47
48 467 *Meat Sci* 2008; 78: 68–76.
49
50
51 468 19. Pérez-Rodríguez ML, Bosch-Bosch N, García-Mata M. Monitoring nitrite and nitrate
52
53 469 residues in frankfurters during processing and storage. *Meat Sci* 1996; 44: 65–73.
54
55
56
57
58
59
60

- 1
2
3
4 470 20. Woolford G, Cassens RG. The fate of sodium nitrite in bacon. *J Food Sci* 1977; 42: 586–589.
5
6
7 471 21. Knowles ME, Gilbert J, McWeeny DJ. Phenols in smoked cured meats. Phenolic
8
9 472 composition of commercial liquid smoke preparations and derived bacon. *J Sci Food Agric*
10
11 473 1975; 26: 189–196.
12
13
14 474 22. Sikorski ZE, Kołakowski E. Smoking. In: Toldrá F (ed) *Handbook of Meat Processing*.
15
16 475 Oxford, UK: Wiley-Blackwell, pp. 231–245.
17
18
19 476 23. Chen BH, Wang CY, Chiu CP. Evaluation of analysis of polycyclic aromatic hydrocarbons
20
21 477 in meat products by liquid chromatography. *J Agric Food Chem* 1996; 44: 2244–2251.
22
23
24 478 24. Sun J, He H, Xie BJ. Novel antioxidant peptides from fermented mushroom *Ganoderma*
25
26 479 *lucidum*. *J Agric Food Chem* 2004; 52: 6646–6652.
27
28
29 480 25. Li X, Han L, Chen L. In vitro antioxidant activity of protein hydrolysates prepared from corn
30
31 481 gluten meal. *J Sci Food Agric* 2008; 88: 1660–1666.
32
33
34 482 26. Sarmadi BH, Ismail A. Antioxidative peptides from food proteins: A review. *Peptides* 2010;
35
36 483 31: 1949–1956.
37
38
39 484 27. de Castro RJS, Sato HH. Biologically active peptides: Processes for their generation,
40
41 485 purification and identification and applications as natural additives in the food and
42
43 486 pharmaceutical industries. *Food Res Int* 2015; 74: 185–198.
44
45
46
47 487 28. Floegel A, Kim D-O, Chung S-J, et al. Comparison of ABTS/DPPH assays to measure
48
49 488 antioxidant capacity in popular antioxidant-rich US foods. *J Food Compos Anal* 2011; 24:
50
51 489 1043–1048.
52
53
54 490 29. Re R, Pellegrini N, Proteggente A, et al. Antioxidant activity applying an improved ABTS

1
2
3
4 491 radical cation decolorization assay. *Free Radic Biol Med* 1999; 26: 1231–1237.
5
6
7 492 30. Damgaard TD, Otte JAH, Meinert L, et al. Antioxidant capacity of hydrolyzed porcine
8
9 493 tissues. *Food Sci Nutr* 2014; 2: 282–288.
10
11
12 494 31. Morrissey PA, Tichivangana JZ. The antioxidant activities of nitrite and nitrosylmyoglobin
13
14 495 in cooked meats. *Meat Sci* 1985; 14: 175–190.
15
16
17 496 32. Mac Donald B, Gray JI, Gibbins LN. Role of nitrite in cured meat flavor: Antioxidant role of
18
19 497 nitrite. *J Food Sci* 1980; 45: 893–897.
20
21
22 498 33. Sebranek JG. Basic Curing Ingredients. In: Tarte R (ed) *Ingredients in Meat Products*. New
23
24 499 York, NY: Springer New York, pp. 1–23.
25
26
27 500 34. Wilkinson BHP, Lee E, Purchas RW, et al. The retention and recovery of amino acids from
28
29 501 pork longissimus muscle following cooking to either 60°C or 75°C. *Meat Sci* 2014; 96: 361–
30
31 502 365.
32
33
34 503 35. National Food Institute. Frida Food Data, ver. 3 <http://frida.fooddata.dk> (2016, accessed 29
35
36 504 August 2017).
37
38
39 505 36. Mora L, Reig M, Toldrá F. Bioactive peptides generated from meat industry by-products.
40
41 506 *Food Res Int* 2014; 65: 344–349.
42
43
44 507 37. Wiechelman KJ, Braun RD, Fitzpatrick JD. Investigation of the bicinchoninic acid protein
45
46 508 assay: Identification of the groups responsible for color formation. *Anal Biochem* 1988; 175:
47
48 509 231–237.
49
50
51
52 510 38. ThermoFisher Scientific. Chemistry of Protein
53
54 511 Assays <https://www.thermofisher.com/dk/en/home/life-science/protein-biology/protein->
55
56
57
58
59
60

1
2
3
4 512 biology-learning-center/protein-biology-resource-library/pierce-protein-methods/chemistry-
5
6 513 protein-assays.html (accessed 1 June 2017).
7
8
9 514
10
11
12 515
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Figure caption

Figure 1. Decline in nitrite over time of storage measured in days in the sandwich ham (SH), the boiled dinner ham (DH) and the smoked pork saddle (SPS).

Figure 2. Aligned representative SEC chromatograms of the aqueous ≤ 10 kDa extracts of the three nitrite-cured cooked ham products after 37 days of storage. For the sandwich ham, SH (top panel), the dinner ham, DH (middle panel) and the smoked pork saddle, SPS (bottom panel) 100 μ L, 200 μ L and 100 μ L extract was injected, respectively. The elution of the injected external standards (peptides and amino acids) is marked on the top panel.

Figure 3. Antioxidant behavior of SEC fractions measure using *in vitro* assays for reducing power (A), and ABTS radical scavenging after 1 min (B) and 31 min (C) incubation.

Figure 4. The content of the total (A) and free (B) amino acids in μ g/mL extract in the ≤ 10 kDa aqueous extracts of the sandwich ham (SH), the one dinner ham (DH) and the smoked pork saddle (SPS).

Tables

Table 1. Antioxidant activity in active peak fractions normalized to protein content in mg. Values in the same column follow by different letters are significantly different ($p < 0.05$).

	Protein specific reducing power (OD ₇₀₀ / mg protein)		Protein specific ABTS radical scavenging (% ABTS radical scavenging activity/mg protein)		
	P1/P4	P2	P1/P4 (31min)	P3 (1min)	P3 (31min)
Sandwich ham (SH)	32.4±8.3 ^a	68.0±4.9 ^a	21760±373 ^a	21827±315 ^a	30557±889 ^a
Dinner ham (DH)	16.6±5.8 ^b	157.6±10.1 ^b	20524±513 ^a	24761±1709 ^b	32031±1637 ^a
Smoked pork saddle (SPS)	24.3±3.2 ^{ab}	73.0±17.5 ^a	10048±751 ^b	35655±694 ^c	51170±1696 ^b

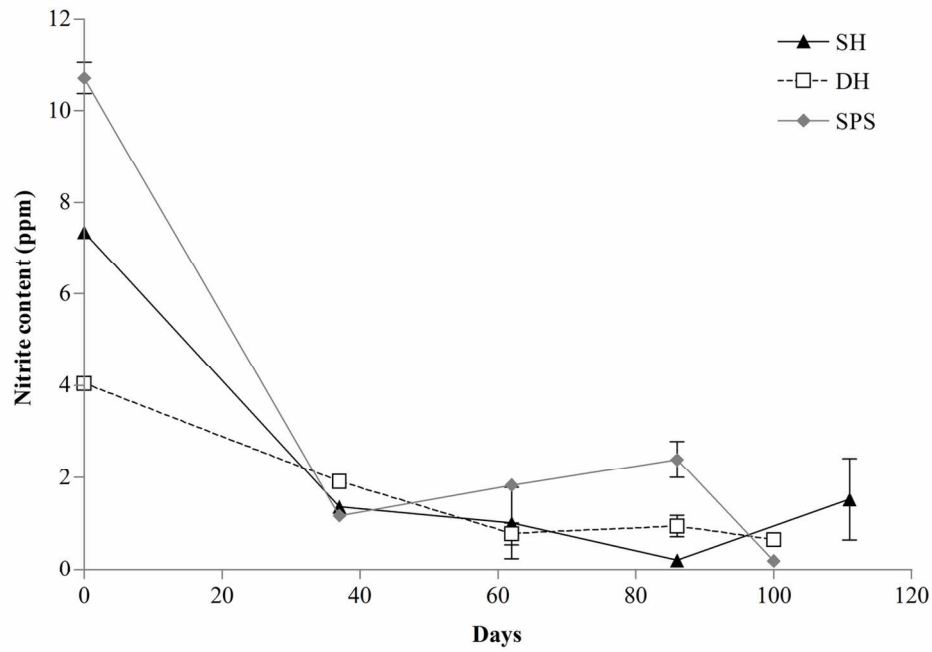
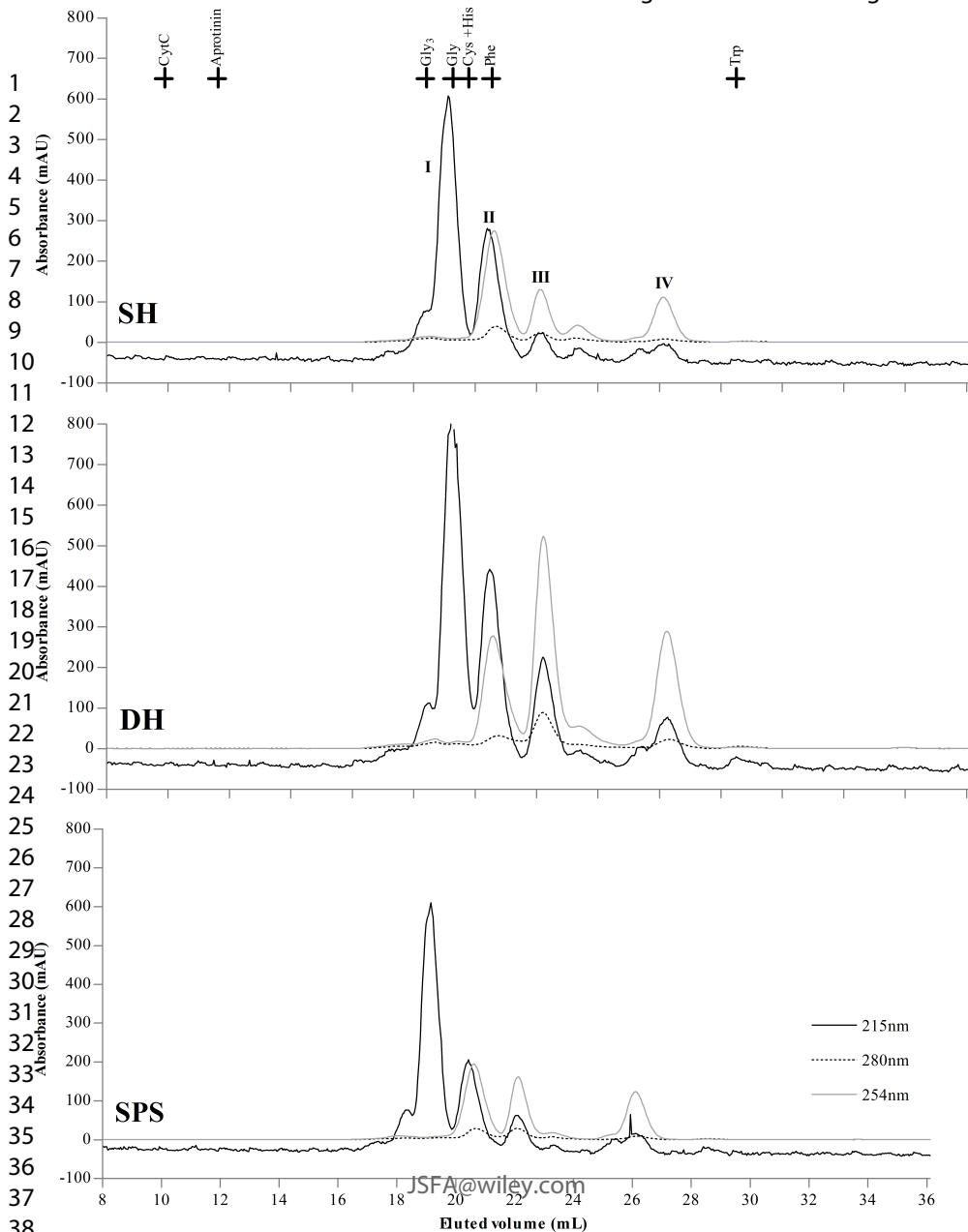
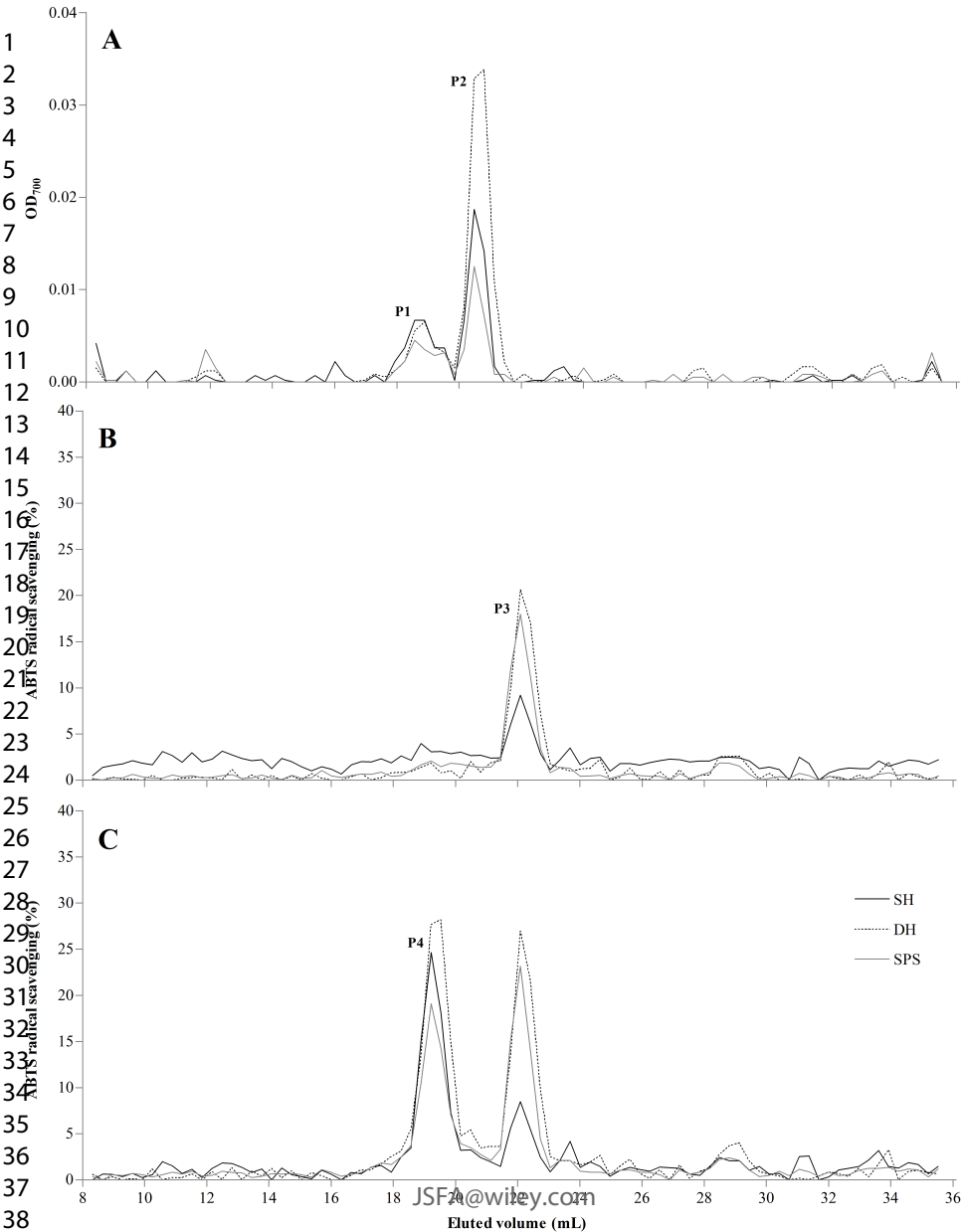


Figure 1

64x46mm (600 x 600 DPI)





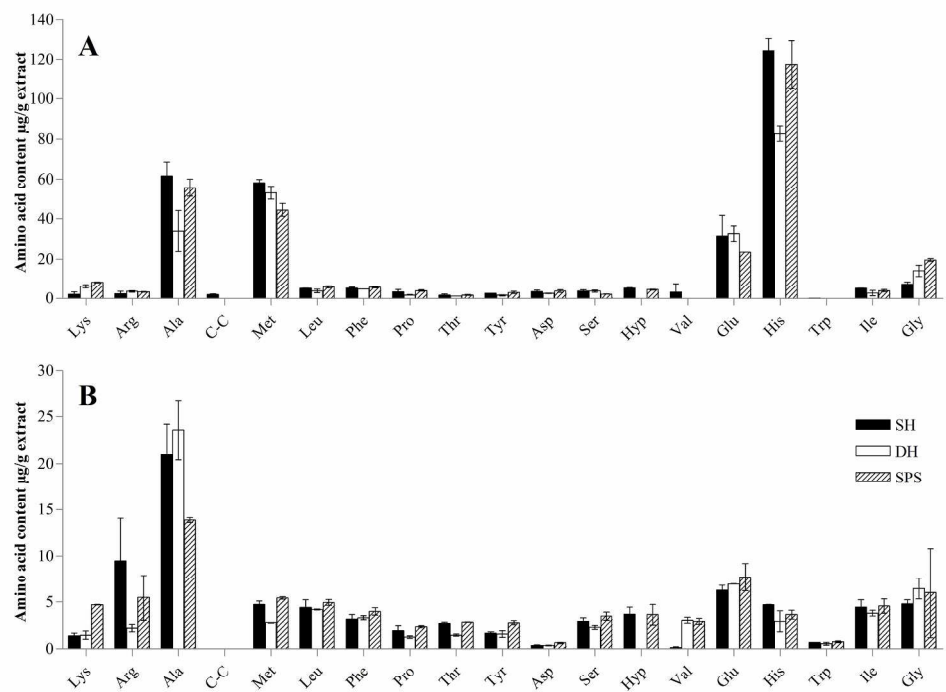


Figure 4

122x107mm (600 x 600 DPI)

Paper III

Pedersen, S. T., Duedahl-Olesen, L., Koch, A. G. & Jessen, F. Effect of storage and varying nitrite and ascorbate concentrations on antioxidative and antimicrobial activity in low molecular weight extracts from nitrite-cured cooked pork products

Title: Effect of storage and varying nitrite and ascorbate concentrations on antioxidative and antimicrobial activity in low molecular weight extracts from nitrite-cured cooked pork products

Authors: Sabrine Tauber Pedersen^a, Lene Duedahl-Olesen^a, Anette Granly Koch^b and Flemming Jessen^a

^aNational Food Institute, Technical University of Denmark, Søtofts Plads Building 227, DK-2800 Kongens Lyngby, Denmark

^bDanish Meat Research Institute, Gregersensvej 9, DK-2630 Taastrup, Denmark

Running title: Effect of nitrite on bioactivity in extracts from nitrite-cured hams

***Corresponding author:**

Sabrine Tauber Pedersen

Postal address:

National Food Institute

Technical University of Denmark

Søtofts Plads Building 227, 1st floor, room 142

DK-2800 Kgs. Lyngby

E-mail:

stape@food.dtu.dk

Declarations of interest: none

23 Abstract

24 For future optimisation of nitrite usage in meat curing this study focused on the influence of nitrite and
 25 ascorbate concentrations on antioxidative and antimicrobial activities. Aqueous ≤ 10 kDa extracts of nitrite-
 26 cured cooked hams produced with different nitrite and ascorbate concentrations were analysed by *in vitro*
 27 assays. ABTS radical scavenging activity increased with added nitrite/ascorbate and with storage time while,
 28 extract reducing power increased with storage time (more pronounced for higher nitrite/ascorbate addition)
 29 and with added nitrite/ascorbate after 12days of cold storage. Iron chelating activity increased with storage
 30 time for extracts of hams added ≥ 150 ppm nitrite and ≥ 600 ppm ascorbate. Antimicrobial activity was not
 31 found. Finally, the ABTS activity increase seemed to be indirectly related to residual nitrite through
 32 formation of NO and the exhibited reducing power appeared partly to be related to residual ascorbate. Iron
 33 chelating activity may in the current situation originate from ham components or a compound formed during
 34 storage.

35

36

37

38 **Keywords:** Cooked hams, curing, nitrite, ascorbate, antioxidant, *in vitro* assays

39

1 Introduction

Preserving of meat by nitrite-curing is a widely used strategy to extend shelf-life as well as creating a microbiologically safe and appealing product. However, utilization of nitrites in food is strictly controlled and limited by current EU legislations. In order to optimise and preferably to further minimise nitrite addition to meat, it is necessary to acquire further knowledge on the protective behaviour of nitrite and other additives in nitrite-cured products.

Although nitrite, as a natural electron acceptor, can be considered a pro-oxidant its use in meat curing is also for antioxidative purposes and nitrite has been pointed out to be a key-responsible not only for the microbiological but also the oxidative stability of cured meat products. Nitrite exerts its antioxidant effect through several different mechanisms (Freyblyer et al., 1993). Yet, nitric oxide (NO) derived from the nitrite ion is more and more accepted as the species interacting with free radicals of lipid oxidation (Skibsted, 2011). Furthermore, NO is essential in the reactions/reactivity of nitrite as it will react with a wide variety of compounds like myoglobin, other proteins and amino acids. Ascorbate is commonly used in combination with nitrite in meat curing. Ascorbate, as an electron donor, is in itself a strong antioxidant that through its relatively unreactive oxidised form (dehydroascorbic acid) can terminate propagation of free radical reactions (Bendich, Machlin, Scandurra, Burton, & Wayner, 1986). Nevertheless, ascorbate may in the presence of metal ions also act as a pro-oxidant (Villaverde, Parra, & Estévez, 2014).

The reaction patterns of nitrite and ascorbate recurrently cross each other e.g. as ascorbate can accelerate the curing process through formation of more NO (Møller & Skibsted, 2002; Skibsted, 2011). Furthermore, ascorbate is consuming oxygen for its own oxidation and thereby limiting oxidation of NO and NO_2^- (Honikel, 2008) and also nitrite reaction products and ascorbate directly interacts forming NO-Asc reaction intermediate (Izumi, Cassens, & Greaser, 1989). During cooking and storage, residual nitrite declines to 10-35% of the originally added amounts (Honikel, 2008; Hustad et al., 1973) which to a certain extent may be explained by the type of reaction mentioned above. Although the redox chemistry of nitrite and ascorbate is essential in nitrite-curing it is highly complex and at times not fully understood.

In the recent years there has been an increased interest in natural antioxidative and antimicrobial compounds, especially to replace unwanted E numbers and preservatives such as nitrite. This has led to an intensification of the search for peptides with antioxidative and antimicrobial properties. Such peptides have been detected in many foods and food products (de Castro & Sato, 2015) however to our knowledge none or remarkably few investigations have been performed on nitrite-cured cooked meat products. Neither has it been possible to come up with a single non-protein alternative to cover all the properties of the multifaceted nitrite (Sindelar & Milkowski, 2011).

All this and a strong interest from the Danish meat industry to use the lowest possible amount of nitrite requires better knowledge about the metabolism of nitrite in meat products. Therefore, it was decided to investigate the effect of ingoing nitrite and ascorbate on antioxidant and antimicrobial activity in nitrite-cured cooked pork products. The levels required to obtain oxidative and microbiological stability in cured meat products to a satisfying degree have previously been examined; however, the oxidative stability has to our knowledge only been assessed by measurements of oxidation products. Thus, it is suggested that this issue by means of antioxidant activity assessment could contribute with a new angle to this field of research. In addition to expecting an effect of amounts of added nitrite/ascorbate it was supposed that cold storage of the hams would result in a decrease in antioxidant capacity with time.

2 Materials and method

2.1 Materials

Ascorbic acid, sodium nitrite, 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-4',4''-disulfonic acid sodium salt (ferrozine) and 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), were acquired from Sigma-Aldrich (St. Louis, MO, USA). Ethylenediaminetetraacetic acid disodium dihydrate (EDTA) was from Merck Millipore (Billerica, MA, USA) and (\pm)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) from Fluka Chemika (Deisenhofen, Germany). All remaining chemicals and

reagents employed were of analytical grade and acquired from VWR International (Radnor, PA, USA), Merck Millipore, Sigma-Aldrich or Fluka Chemika.

2.2 Samples

Hams belonging to the category of restructured meat products where smaller pieces of meat are subjected to curing brine prior to boiling stuffed into a plastic casing. The main recipe constituted 75% pork meat, 20% water, 2.1% salt, skin protein (*Danish: sværprotein*), phosphate, potato starch and dextrose with a final water content of approximately 75%. The main recipe was identical for all hams only changing the added amount of sodium nitrite and ascorbate. The final concentrations were 0, 60, 150, 250, 500 and 1000ppm of added sodium nitrite and 4 times as much ascorbate. The individual hams will be referred to as added sodium nitrite slash added sodium ascorbate e.g. 60/240. The meat was vacuum packed and stored at -80°C until analysis.

A storage experiment was carried out where the sample were thawed and left at 5°C for a total of one month periodically taking out samples and returning them to -80°C until further analysis.

2.3 Extraction of low molecular weight compounds

Water soluble compounds were extracted from the cured hams by homogenising approximately 70-100 g of minced ham in approximately 400mL of deionised water, dH₂O, for 1 minutes with an Ultra Turrax (T 25 Basic ULTRA-TURRAX® mounted with S25N -18G Dispersing element, IKA®-WERKE GmbH &Co. KG, Staufen, Germany) at 13,500rpm and subsequently adding up to a total volume 1.5L. An aqueous ≤10kDa extract was collected by submerging three to five pierces of dialysis membrane tube of 10 cm each (Spectra/Por 6, MWCO 10,000 Da, Spectrum Lab. Inc, CA, USA) containing 16.5mL dH₂O (corresponding to 50% membrane volume) into the meat/water homogenate. After 24 hours of magnetic stirring at 5°C, the dialysate in the membranes were pooled and stored at -80°C until further analysis. Later the protein content was determined colourimetrically using the Pierce™ Modified Lowry Protein Assay Kit in microplate format according to manufactures description (Thermo Scientific, Pierce™) using Bovine Serum Albumin (BSA) (Albumin Standard Ampules, 2 mg/ml) supplied with the kit as an external standard.

113 2.4 Nitrite content

114 The sodium nitrite content was determined in both the hams and the low molecular weight extracts hereof.
115 The day before analysis, approximate 50g of meat sample stored at -80°C was thawed, minced for ten
116 seconds, and re-vacuum packed before being returned to -80 °C. Extraction and measurement of nitrite in the
117 cured cooked hams was done according to the method for simultaneous measurement of nitrite and nitrate
118 described by Herrmann, Duedahl-Olesen, & Granby (2015). The detection was carried out using ion
119 chromatography coupled to UV detection at 225nm (HPLC and Diode Array Detector, series1100, Agilent
120 Technologies, Santa Clara, CA, USA) with separation on a Dionex IonPac™ AS11 RFICTM 250 x 4mm
121 (Thermo Scientific, Waltham, MA, USA). The extracts were diluted 1:1(V/V) in 1mM NaOH to obtain a pH
122 comparable to mixed minced ham and 1mM NaOH (1+10). The subsequent extraction, clean-up and analysis
123 were identical for the two sample types. The samples were analysed in a random order.

124 2.5 Ascorbic acid

125 Residual ascorbic acid in the hams and extracts was determined using an Agilent 1100 series HPLC using a
126 G1315A DAD detector set at 254.4nm (Agilent Technology, Waldbronn, Germany).
127 Ascorbic acid was extracted by leaving 2.5g of minced ham and 10mL of 5%(W/V) meta-phosphoric acid,
128 added 0.5mL of 0.1mg/mL EDTA solution, to stir for 2 minutes at room temperature protected from light.
129 MilliQ was added to a total volume of 25mL. The mixture was centrifuged at 3000rpm for 5 minutes and
130 filtered through a Munktell quantitative filter paper grade 00H and re-filtered through a 0.2µm syringe filter
131 directly into an amber HPLC vial. The extracts were merely mixed 1:1 with 1% (W/V) meta-phosphoric acid
132 and filtered through a 0.2µm syringe filter directly into an amber HPLC vial. Twenty µL sample was injected
133 and separated on a Prodigy 5u ODS3 100Å 250x4.6mm column (Phenomenex®, Torrance, CA, USA) and
134 eluted at a flow rate of 0.7mL/min. Mobile phase A was water adjusted to pH 3 and B was methanol running
135 a gradient of 5% B for the first 23 min followed by a 10second linear increase to 100% B which were kept
136 for 4min and 50 seconds before again decreasing to 5% B in 10 seconds and kept at this level until the end of
137 the run (total of 35 min). Quantification was done using an external calibration curve of L-ascorbic acid
138 dissolved in 1% (W/V) meta-phosphoric acid. The samples were analysed in a random order.

2.6 Antioxidant assays

Potential antioxidant activities of the aqueous ≤ 10 kDa extract were analysed using three *in vitro* assays measuring iron chelation activity, reducing power and ABTS-radical scavenging activity. In order to investigate the concentration dependency of the antioxidant activities, the crude undiluted ≤ 10 kDa extract were analysed followed by sequential threefold dilutions. The assays were conducted in triplicate and all measurements were performed using a spectrophotometer (Synergy 2 Multi-Mode Microplate Reader, BioTek® Instruments, Inc., Vermont, USA). The extracts were thawed at 5°C, over night.

Iron chelating activity was assayed by the microtiter plate format method described by Hermund et al. (2015). EDTA (0.5 mM) was used as a positive control and was subjected to the same threefold dilutions as the extract samples. dH₂O was utilised as blank. The iron chelating activity (%) is defined and calculated as percentage of inhibited colour formation/relative decrease in absorbance (562 nm):

$$Fe^{2+} \text{ chelating activity (\%)} = \frac{Abs_{blank} - Abs_{sample}}{Abs_{blank}} \cdot 100\% \quad (1)$$

150

The reducing power was determined according to the procedure described by Oyaizu (1986) with some modifications and adjusted to microtiter plate detection. First, equal parts of ≤ 10 kDa extract sample (and dilutions), 0.2 M phosphate buffer (pH 6.6) and 1% potassium ferricyanide were mixed and incubated for 20 min at 50°C. Subsequently, 1 part of 10% TCA was also added. Then, 100 μ L of the sample mixture was mixed with 100 μ L dH₂O and 20 μ L of 0.1% ferric chloride, and incubated 10 minutes at room temperature. The absorbance was measured at 700 nm. Ascorbic acid (0.5 mM) was used as positive control and was subjected to the same threefold dilution as the extract samples and dH₂O was used as blank. Results are expressed as

$$OD_{700} = Abs_{700nm}(Sample) - Abs_{700nm}(blank) \quad (2)$$

159

The radical scavenging activity was determined according to the method described by Gringer, Osman, Nielsen, Undeland, & Baron, (2014). Trolox (2.5mM, in 96% ethanol) was used as a positive control and was subjected to the same threefold dilutions as the samples. Absorbance was measured after 8 minutes. Ethanol (96%) and dH₂O was included as blank for the positive control and extract sample, respectively. The ABTS radical scavenging activity (%) for the individual dilution is expressed as the relative decrease in absorbance (734nm) as for iron chelating activity

$$ABTS \text{ radical scavenging activity (\%)} = \frac{Abs_{blank} - Abs_{sample}}{Abs_{blank}} \cdot 100\% \quad (3)$$

As all the crude ≤ 10 kDa extracts and some of the dilutions with highest extract concentration reached the assays maximal capacity around 90%, similar to the positive control (Trolox), the ABTS radical scavenging activity of the extracts was expressed as normalised to ham (%/mg ham) and denoted as Normalised ABTS radical scavenging activity. This was calculated as the initial linear slope from curves of ABTS radical scavenging activity (%), as expressed in equation (3), versus mg ham constituting the basis for the individual dilutions.

2.7 Antimicrobial activity

Antimicrobial activity of the cured ham aqueous ≤ 10 kDa extracts and the supernatant of the meat/water homogenate were tested with an inoculation culture consisting of a mixture of *Listeria monocytogenes* (DMRICC 3042) and *Salmonella typhimurium* (DMRICC 4984). A small quantity of the purified cultures where each transferred to 10mL Brain-heart infusion (BHI) broth (Oxoid CM1135, United Kingdom) and incubated for 20 ± 2 hours at 37°C. These were afterwards diluted to 10⁻⁶ and mixed 1:1 (V/V) to constitute the inoculation culture.

Diluted (1:5 and 1:25) and undiluted extracts were mixed 1:1 with BHI broth and subsequently inoculated with the inoculation culture. The initial theoretical concentration of each bacteria culture in the inoculated samples is 44 cfu/ml ~ 1.6 log cfu/ml. A sample substituting extract with FKP (0.85% NaCl (Merck 1.06404, Germany) and 0.1% pepton (Merck 1.07214, Germany)) was included as a control. The inoculated extract samples were incubated at 17 ± 1 °C and samples were taken after 0, 24, 48 and 72 hours. The total bacterial

count was determined on Oxford agar (oxoid CM 0856, United Kingdom) and SSI Enteric agar (34121, Statens Serum Institute, Copenhagen Denmark) after 24-48 hours of incubation at 37°C.

2.8 Statistical analysis

All analyses were carried out in triplicate unless otherwise stated. Statistical analyses were conducted using the GraphPad Prism® software Ver. 4.03 or R version 3.3.0 with $P < 0.05$. Results were compared using one-way ANOVA and t-test with Tukeys posttest. Regression analyses were conducted in excel using the regression function in the Data analysis toolbox.

3 Results

3.1 Antioxidant activity

Preliminary experiments (data not shown) had indicated a potential effect of storage time on the investigated types of *in vitro* antioxidant activity in extract from cured hams. Thus, a storage experiment was set up to evaluate the impact of time, as well as nitrite/ascorbate addition, on *in vitro* iron chelating activity, reducing power and ABTS radical scavenging activity during 30 days of cold storage. The protein content of the aqueous ≤ 10 kDa extracts of the hams stored for 0-30 days and taken out with 3 days intervals was on average 0.25 ± 0.06 mg/mL and was not significantly different between hams of different nitrite/ascorbate addition and neither between days of storage.

3.1.1 Iron chelating activity

The iron chelating activity of the crude ≤ 10 kDa extracts ranged from approximately 36-88% with 250/1000 showing the highest average activity across time and 1000/4000 the lowest. In comparison the positive control in the tested concentration (0.5mM EDTA) demonstrated approximately 95% iron chelating activity. The average iron chelating activity across storage time of the remaining crude ≤ 10 kDa extract arranged in the order $0/0 > 60/240 > 500/2000 > 150/600$.

No apparent correlation between iron chelating activity in the crude ≤ 10 kDa extracts and storage time was evident across all nitrite/ascorbate concentrations (Figure 1). However, a significantly (regression analysis) increasing iron chelating activity of the crude ≤ 10 kDa extracts over time was found in sample 150/600, 250/1000, 500/2000 and 1000/4000.

3.1.2 Reducing power

Figure 2 shows the reducing power of the crude ≤ 10 kDa extracts as a function of storage time. At day 0 the order of reducing power activity was 150/600 > 60/240 > 0/0 > 250/1000 > 500/2000 > 1000/4000. The analysis OD_{700} was lower in the extract samples compared to the positive control (0.5mM ascorbic acid) which exhibited an OD_{700} of 0.72 ± 0.05 . Looking at the analytical dilution curves of the samples from day 0 (Figure 3) the reducing power activity increased continuously with the extract concentration for samples 0/0, 60/240 and 150/600 (Figure 3). In contrast the activity of the samples 250/1000, 500/2000 and 1000/4000 only increased with concentration at low extract concentrations however decreased at higher extract concentrations to an extent where no reducing power were demonstrated in the crude ≤ 10 kDa extract of the sample added the most nitrite and ascorbate (1000/4000). However, after 3 days of storage the reducing power activity of the 1000/4000, 500/2000 and 250/1000 crude ≤ 10 kDa extracts started to surpass the reducing power activity of the three low nitrite/ascorbate samples (Figure 2). And after 12 days of cold storage the measured extract reducing power activities directly correlated with added nitrite/ascorbate. For all samples there were a significant (regression analysis) positive correlation between reducing power and storage time (Figure 2).

Regardless of the decrease in reducing power detected early in storage for samples of high extract concentration and high nitrite/ascorbate addition the initial linear reducing power activity as function of mg ham increased with the added nitrite/ascorbate for all samples (Figure 4). However, there were no significant and consistently positive correlation between the initial linear reducing power activity and time of storage (Figure 4).

3.1.3 ABTS radical scavenging

The normalised ABTS radical scavenging activity increased significantly (regression analysis) for all extracts both with storage time and with increased nitrite/ascorbate addition (Figure 5). As an example, it may be mentioned that the ABTS radical scavenging activity at day 0 was approximately 3.5 times higher for 1000/4000 compared to 0/0.

3.2 Antimicrobial activity at day 0

In the tested concentrations, neither growth of *Listeria monocytogenes* nor *Salmonella typhimurium* were inhibited by any of the day 0 ≤ 10 kDa extracts compared to the control (ham extract substituted for equivalent amounts diluent). This was the case after 0, 24, 48 and 72 hours of incubation. Testing the supernatants of the meat/water homogenates used for producing the ≤ 10 kDa extracts gave the same result.

3.3 Residual ascorbic acid and nitrite

Residual ascorbic acid was measured in the ≤ 10 kDa extracts of hams stored for 0, 3, 6, 12, and 21 days at 5°C. This was normalised to the amount of ham utilised for the ham/water homogenate used for producing the extracts and is presented in Figure 6. Residual ascorbic acid increased with added nitrite/ascorbate for the samples 0/0, 60/240 and 150/600 and remained at the same level during the 21 days of storage (0/0: 0.06-0.07 $\mu\text{g/g}$ ham; 60/240: 0.4-0.7 $\mu\text{g/g}$ ham; 150/600: 1.1-1.3 $\mu\text{g/g}$ ham) with the exception of slight drop for sample 150/600 at day 21. At day 0 the residual ascorbic acid content in the three remaining extract samples were lower than the 150/600 sample and decreased with increasing added nitrite/ascorbate. A similar pattern was also evident for residual ascorbic acid in the hams at day 0 (data not shown). At day 12 extract samples 250/1000 and 500/2000 had increased to a level significantly different from the previous measurement days, and now these two samples also demonstrated higher residual ascorbic acid content than the three samples with the lowest added nitrite/ascorbate concentrations. At day 21 the residual ascorbic acid content correlated with added nitrite/ascorbate for all samples. Detectable residual ascorbic acid increased approximately 55 times in extract 1000/4000 during the 21 days of cold storage

Residual sodium nitrite was measured in the ≤ 10 kDa extracts of hams stored for 0, 3, 6, 12, 21 and 30 days at 5°C. This was also normalised to the amount of ham utilised for the ham/water homogenate used for producing the extracts and is presented in Figure 7. Residual sodium nitrite correlated with added sodium nitrite throughout storage yet the content generally decreased with time (no significant decrease was found for 0/0 and 60/240 at any time points). Residual sodium nitrite was also measured in the hams at day 0 (data not shown) and here residual and added sodium nitrite also correlated. The hams contained on average approximately 18 times more nitrite per g than the extracts contained per mL – matching the ratio between minced ham and total volume of 1.5L for the meat/water homogenate (Materials and Method).

4 Discussion

4.1 Reducing power

The surprising shape difference in the reducing power dilution curves of sample extract 0/0, 60/240 and 150/600 versus 250/1000, 500/2000 and 1000/4000 (Figure 3) at day 0 indicated a clear concentration dependence. This furthermore indicated that at higher concentrations the active reducing components in the ≤ 10 kDa extracts potentially resulted in an overload of the assay and that this was somehow related to addition of higher concentrations of nitrite/ascorbate. The applied assay for determining *in vitro* reducing power was based on the formation of pigment Prussian blue ($\text{Fe}^{\text{III}}[\text{Fe}^{\text{III}}\text{Fe}^{\text{II}}(\text{CN})_6]_3$) that is formed upon reduction of potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}) by an antioxidant present in the sample in the presence of excess ferric chloride (Fe^{3+}) (Wiberg, Wiberg, & Holleman, 2001). However, the electrochromic nature of the Prussian blue pigment may cause it to return to colourless upon further reduction of Fe^{3+} to Fe^{2+} . The large amounts of ascorbic acid added to the three samples in question could have caused this double reduction however this explanation is contradictive to the amount of residual ascorbic acid in the samples. On the other hand, the inter-sample pattern of reducing power activity of the crude ≤ 10 kDa extracts at day 0 showed some resemblance with the residual ascorbic acid in regard to the drop occurring at additive concentration above 150/600. However, the residual ascorbic acid content of sample 500/2000 and 1000/4000 were almost as low as 0/0 yet this did not transfer trough to the reducing

power determined in crude 0/0. The relationship between residual ascorbic acid and reducing power of the crude ≤ 10 kDa extracts continued during storage with a slight time discrepancy. At day 12 the reducing power activity increased with increasing nitrite/ascorbate addition while residual ascorbic acid did not increase with added nitrite/ascorbate until day 21. However the full change to a residual/added ascorbic acid correlation might have occurred just after day 12 (only 1000/4000 did not match the correlation at this point) and then have become more pronounced from thereon as was also the case with the reducing power.

285

286 4.2 Ascorbic acid

Plant foodstuff e.g. fruits and vegetables are the primary source of dietary ascorbic acid. Nutrient data on fresh pork meat rarely reports of any ascorbic acid content at all. However, ascorbic acid is found in all living cells, plant as well as animal, which may explain the minute amounts found in the ham and extract of the 0/0 sample (ham: approx. 13ppm ($\mu\text{g/g}$ WW). Extracts: approx. $1\mu\text{g/mL}$) (Belitz, Grosch, & Schieberle, 2004). Otherwise the detected residual ascorbic acid in the remaining samples must be assumed to originate from the added sodium ascorbate. However, only approximately half of the sodium ascorbate added to the hams was recovered as residual ascorbic acid (sample 60/240 and 150/600) which match the levels of residual ascorbic acids found in other commercial processed meat products (Hidiroglou, Madere, & Behrens, 1998; Schüep & Keck, 1990; Valls, Sancho, Fernández-Muiño, Alonso-Torre, & Checa, 2002). Ascorbic acid is readily (and reversible) oxidised to dehydroascorbic acid but may also be further degraded by opening of the ring-structure leading to decrease or loss, respectively, of antioxidant activity. This oxidation and degradation depends on a number of factors in particular oxygen partial pressure, pH, temperature and presence of heavy metal ions e.g. Cu^{2+} and Fe^{3+} (Belitz et al., 2004). Essential in regard to the loss of measurable ascorbic acid in the cured hams is ascorbic acid's interactions/reactions with the added nitrite leading to its oxidation but also formation of nitrosated reaction intermediates (capable of functioning as nitrosating agents) – intermediates most likely not detected by the applied HPLC method [Reactions of nitrite and ascorbate are reviewed in Skibsted (2011) and Honikel, (2008)]. The EDTA added to the samples during sample preparation may have protected and stabilised any residual ascorbic acid from oxidizing but as

a metal chelator it is not considered likely that it has had an impact on such intermediates or their formation. The many ways in which ascorbic acid may be degraded or react with other ham components does however not offer a clear explanation for the lower ascorbic acid detected in the three concentrated samples and not the samples 0/0, 60/240 and 150/600 and neither does it provide an explanation for the increase over time.

4.3 Iron chelating activity

Iron chelating activity was detected in all tested ≤ 10 kDa sample extracts throughout storage yet there did not appear to be any correlation with amounts of nitrite/ascorbate initially added to the hams. This being in spite of ascorbic acid being capable of chelating metals (Brady, 2013) and some of the proposed antioxidant activities of nitrite involve chelating of metals (Igene, Yamauchi, Pearson, Gray, & Aust, 1985). A similar lack of correlation also came from the investigations of a small selection of commercial cooked cured hams of known nitrite addition concentration (Pedersen et al., n.d.). Furthermore, results did neither correlate with residual amounts of sodium nitrite or ascorbic acid. Thus, it must be assumed that the detected activity originated from other ham components in the extracts. Some phosphates are known to function as metal chelators and could as such be expected to also contribute to the iron chelating activity signals (Igene et al., 1985) however the same amount of phosphates were added to all the hams. Therefore the slight differences observed could stem from other sources potentially related to differences in the utilised pork meat e.g. proteins or amino acids rather than additives. Throughout the storage period the activity of extracts 150/600, 250/1000, 500/2000 and 1000/4000 increased yet the lack of a direct correlation with amounts of nitrite/ascorbate initially added to the hams remained. Yet as this tendency was not evident in the two samples of lowest or no added nitrite/ascorbate, this inspired the hypothesis that nitrite/ascorbate addition beyond a certain threshold concentration indirectly affect the time depended formation of the active component(s).

4.4 ABTS radical scavenging

At day 0 the normalised ABTS radical scavenging activity correlated positively with the amount of added nitrite/ascorbate (Figure 5). As added and residual sodium nitrite converged in the current study, the

normalised ABTS radical scavenging activity also correlated with residual sodium nitrite. This correlation was also reported in a previous study (Pedersen et al., n.d.). However, the increase in normalised ABTS radical scavenging activity occurring over time (Figure 5) was contrary to the decrease in residual sodium nitrite happening over time (higher added sodium nitrite still resulted in higher residual sodium nitrite at any time point during storage). This type of decrease in residual nitrite during cold storage has often been reported (Pérez-Rodríguez, Bosch-Bosch, & García-Mata, 1996). As nitrite do not primarily exert its versatile antioxidant properties directly as nitrite but through reaction products like NO and MbNO (Freybler et al., 1993; Honikel, 2008; Skibsted, 2011) it can be assume that the decrease in residual nitrite over time is resulting in formation of more antioxidant species and that a higher addition of nitrite provides more starting material for such a conversion. Thus, the increase in normalised ABTS radical scavenging activity may be linked with added as well as residual nitrite. The correlation between normalised ABST radical scavenging and residual nitrite reported by Pedersen et al. (n.d.) does not fit with this explanation as residual and added nitrite did not correlate. This might partly be due to the nature of the sample material as the hams in the current study are more or less identical with the exception of differences in added amounts of nitrite/ascorbate whereas the samples used by Pedersen et al. (n.d.) were very different (type of processing/cooking, national origin, additives etc.) and lacking details on other additives like amount of added ascorbate. These differences can have affected the conversion of nitrite to its active antioxidant species e.g. higher addition of ascorbate result in faster turnover of nitrite (Izumi, 1992).

Ascorbic acid is generally regarded as a reducing agent but is also occasionally referred to as a radical scavenger (Villaverde, Ventanas, & Estévez, 2014). However, the normalised ABTS radical scavenging activity did not correlate with residual ascorbic acid until the end of the storage period. Furthermore, we have not been able find literature on ascorbic acids ability to scavenge the ABTS radical. As 0/0 also demonstrated an activity although not as high as the nitrite-cured samples, it can be speculated that the curing agents might merely accelerate the ABTS radical scavenging properties naturally enclosed in the hams. The same can be said regarding the initial reducing power activity.

4. 5 Antimicrobial activity

It was decided to investigate whether the antimicrobial activities known from nitrite-cured products was associated with the water-soluble ≤ 10 kDa fraction and most importantly if it was related to the ingoing and residual amount of additives and more specifically sodium nitrite and sodium ascorbate. Despite that nitrite-derived NO is known to be toxic towards *L. monocytogenes* (Cammack et al., 1999) no growth inhibitory effect was evident from the extracts at any additive concentration. Several studies have emphasised the importance of e.g. ingoing and residual nitrite as well as pH and storage temperature (Birzele, Djordjević, & Krämer, 2005; King, Glass, Milkowski, Seman, & Sindelar, 2016; McClure, Kelly, & Roberts, 1991). A relationship between ingoing nitrite and antimicrobial properties through NO makes great sense as more added nitrite logically would give more starting material for NO formation. However, as no effects based on current results can be assigned to ingoing nitrite it could also be considered whether the lack of response is due to a combination of other parameters such as low residual nitrite ($\leq 14.8 \mu\text{g/mL}$ extract equivalent to $0.9 \mu\text{g/g}$ ham used for the extract). Birzele et al. (2005) found *L. monocytogenes* inhibition to correlate with concentration of nitrite in the applied curing salt however the residual nitrite were never below 19mg/kg sample. So in the event that the residual nitrite in their study contributed to the inhibitory effect the lower levels found in the current study may explain the absence of an antimicrobial effect. The lack of a response may potentially also be connected to pH as McClure et al. (1991) found that much higher amounts of ingoing nitrite were required at pH above 5.3 and the pH of the extracts were around 6. Hospital, Hierro, & Fernández (2014) reported that 75ppm ingoing nitrite led to a reduction in *Salmonella* in raw minced pork and sausages during storage however the effect of nitrite on *Salmonella* is a controversial issue and research seems to point in opposite directions. Thus if any antimicrobial effect at all exists the lack of response in the current study (also towards *L. monocytogenes*) must be due to low concentration of the active components in the tested samples.

Conclusion

It was expected that nitrite/ascorbate addition and time of cold storage would affect *in vitro* antioxidant activity. All three tested types of antioxidant activity increased with storage time although not all significantly and not for all samples. Collectively the different types of antioxidant activity are not solely correlated with residual levels of ascorbate and nitrite despite correlations with added nitrite/ascorbate in the case of normalised ABTS radical scavenging and initial linear reducing power activity. The results show that other components than nitrite and ascorbate are of great importance for antioxidative activities in ≤ 10 kDa ham extracts. The identity and nature of these compounds need to be investigated in order to optimise and preferably minimise the addition of additives to cured meat without needlessly compromising product stability. The unexpected pattern detected in residual ascorbic acid during storage, which correlated with *in vitro* reducing power, requires further investigations for an explanation.

Acknowledgment

Hams were kindly manufactured by Danish Meat Research Institute. Furthermore thanks are expressed to Flemming Hansen and Anita Forslund for contributing to idea generation and supervision in all microbiological matters.

This work was supported by a grant from Norma and Frode S. Jacobsens Fond.

References

- Belitz, H.-D., Grosch, W., & Schieberle, P. (2004). *Food Chemistry* (3rd ed.). New York, NY: Springer Berlin Heidelberg.
- Bendich, A., Machlin, L. J., Scandurra, O., Burton, G. W., & Wayner, D. D. M. (1986). The antioxidant role of vitamin C. *Advances in Free Radical Biology & Medicine*, 2(2), 419–444.
- Birzele, B., Djordjević, S., & Krämer, J. (2005). A study of the role of different nitrite concentrations on human pathogenic bacteria in fresh spreadable ham and onion sausage. *Food Control*, 16(8), 695–699.

- 402 Brady, J. W. (2013). *Introductory food chemistry* (1st ed.). Comstock Pub. Associates.
- 403 Cammack, R., Joannou, C. L., Cui, X.-Y., Martinez, C. T., Maraj, S. R., & Hughes, M. N. (1999). Nitrite and
404 nitrosyl compounds in food preservation. *Biochimica Et Biophysica Acta*, 1411(2–3), 475–488.
- 405 de Castro, R. J. S., & Sato, H. H. (2015). Biologically active peptides: Processes for their generation,
406 purification and identification and applications as natural additives in the food and pharmaceutical
407 industries. *Food Research International*, 74, 185–198.
- 408 Freybler, L. A., Gray, J. I., Asghar, A., Booren, A. M., Pearson, A. M., & Buckley, D. J. (1993). Nitrite
409 stabilization of lipids in cured pork. *Meat Science*, 33(1), 85–96.
- 410 Gringer, N., Osman, A., Nielsen, H. H., Undeland, I., & Baron, C. P. (2014). Chemical characterization,
411 antioxidant and enzymatic activity of brines from scandinavian marinated herring products. *Journal of*
412 *Food Processing & Technology*, 5(346).
- 413 Hermund, D. B., Yeşiltaş, B., Honold, P., Jónsdóttir, R., Kristinsson, H. G., & Jacobsen, C. (2015).
414 Characterisation and antioxidant evaluation of Icelandic *F. vesiculosus* extracts in vitro and in fish-oil-
415 enriched milk and mayonnaise. *Journal of Functional Foods*, 19, 828–841.
- 416 Herrmann, S. S., Duedahl-Olesen, L., & Granby, K. (2015). Occurrence of volatile and non-volatile N-
417 nitrosamines in processed meat products and the role of heat treatment. *Food Control*, 48(Sp. Iss. SI),
418 163–169.
- 419 Hidirolou, N., Madere, R., & Behrens, W. (1998). Electrochemical determination of ascorbic acid and
420 isoascorbic acid in ground meat and in processed foods by high pressure liquid chromatography.
421 *Journal of Food Composition and Analysis*, 11, 89–96.
- 422 Honikel, K.-O. (2008). The use and control of nitrate and nitrite for the processing of meat products. *Meat*
423 *Science*, 78(1–2), 68–76.
- 424 Hospital, X. F., Hierro, E., & Fernández, M. (2014). Effect of reducing nitrate and nitrite added to dry
425 fermented sausages on the survival of *Salmonella Typhimurium*. *Food Research International*, 62,
426 410–415.
- 427 Hustad, G. O., Cervený, J. G., Trenk, H., Deibel, R. H., Kautter, D. A., Fazio, T., ... Kolari, O. E. (1973).
428 Effect of sodium nitrite and sodium nitrate on botulinal toxin production and nitrosamine formation in
429 wieners. *Applied Microbiology*, 26(1), 22–26.
- 430 Igene, J. O., Yamauchi, K., Pearson, A. M., Gray, J. I., & Aust, S. D. (1985). Mechanisms by which nitrite

- 431 inhibits the development of warmed-over flavour (WOF) in cured meat. *Food Chemistry*, 18(1), 1–18.
- 432 Izumi, K. (1992). Reaction of nitrite with ascorbic acid or ascorbic acid-2-derivatives. *Journal of Food*
 433 *Science*, 57(5), 1066–1067.
- 434 Izumi, K., Cassens, R. G., & Greaser, M. L. (1989). Reaction of nitrite with ascorbic acid and its significant
 435 role in nitrite-cured food. *Meat Science*, 26(2), 141–153.
- 436 King, A. M., Glass, K. A., Milkowski, A. L., Seman, D. L., & Sindelar, J. J. (2016). Modeling the impact of
 437 ingoing sodium nitrite, sodium ascorbate, and residual nitrite concentrations on growth parameters of
 438 *Listeria monocytogenes* in cooked, cured pork sausage. *Journal of Food Protection*, 79(2), 184–193.
- 439 McClure, P. J., Kelly, T. M., & Roberts, T. A. (1991). The effects of temperature, pH, sodium chloride and
 440 sodium nitrite on the growth of *Listeria monocytogenes*. *International Journal of Food Microbiology*,
 441 14(1), 77–91.
- 442 Møller, J. K. S., & Skibsted, L. H. (2002). Nitric oxide and myoglobins. *Chemical Reviews*, 102(4), 1167–
 443 1178.
- 444 Oyaizu, M. (1986). Studies on products of browning reaction. Antioxidative activities of products of
 445 browning reaction prepared from glucosamine. *The Japanese Journal of Nutrition and Dietetics*, 44(6),
 446 307–315.
- 447 Pedersen, S. T., Baron, C. P., Gobba, C. De, Duedahl-Olesen, L., Koch, A. G., & Jessen, F. (n.d.).
 448 Antioxidative and antimicrobial activity of extracts from nitrite-cured cooked pork products.
 449 *Unpublished manuscript*.
- 450 Pérez-Rodríguez, M. L., Bosch-Bosch, N., & Garcíá-Mata, M. (1996). Monitoring nitrite and nitrate residues
 451 in frankfurters during processing and storage. *Meat Science*, 44(1–2), 65–73.
- 452 Schüep, W., & Keck, E. (1990). Measurement of ascorbic acid and erythorbic acid in processed meat by
 453 HPLC. *Zeitschrift Für Lebensmittel-Untersuchung Und -Forschung*, 191(4–5), 290–292.
- 454 Sindelar, J. J., & Milkowski, A. L. (2011). Sodium nitrite in processed meat and poultry meats: A review of
 455 curing and examining the risk benefit of its use. *AMSA White Paper Series*, 3, 1–14.
- 456 Skibsted, L. H. (2011). Nitric oxide and quality and safety of muscle based foods. *Nitric Oxide*, 24(4), 176–
 457 183.
- 458 Valls, F., Sancho, M. T., Fernández-Muiño, M. A., Alonso-Torre, S., & Checa, M. A. (2002). High-pressure
 459 liquid chromatographic determination of ascorbic acid in cooked sausages. *Journal of Food Protection*,

460 65(11), 1771–1774.

461 Villaverde, A., Parra, V., & Estévez, M. (2014). Oxidative and nitrosative stress induced in myofibrillar
462 proteins by a hydroxyl-radical-generating system: Impact of nitrite and ascorbate. *Journal of*
463 *Agricultural and Food Chemistry*, 62(10), 2158–2164.

464 Villaverde, A., Ventanas, J., & Estévez, M. (2014). Nitrite promotes protein carbonylation and Strecker
465 aldehyde formation in experimental fermented sausages: Are both events connected? *Meat Science*,
466 98(4), 665–672.

467 Wiberg, E., Wiberg, N., & Holleman, A. F. (Arnold F. (2001). *Inorganic chemistry*. London: Academic
468 Press.

469

470

Figures

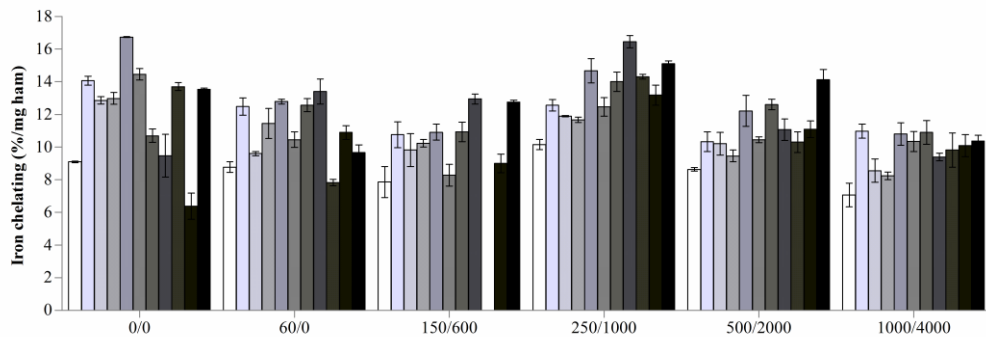


Figure 1 Average iron chelating activity (%/mg ham) in the crude ≤ 10 kDa extracts during storage distributed on ham type. The white bar indicates 0 days of cold storage and for each shade darker +3 days of storage until the black bar indicating 30 days of cold storage. Sample 150/600 at day 24 was lost.

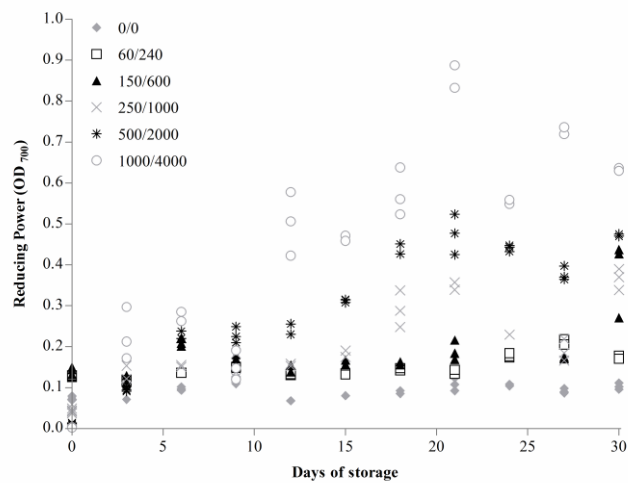
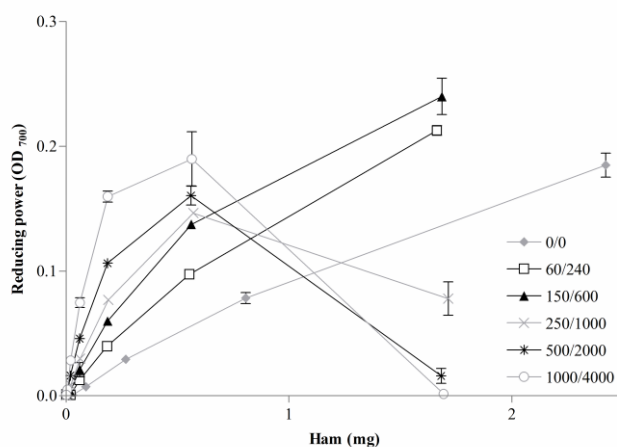


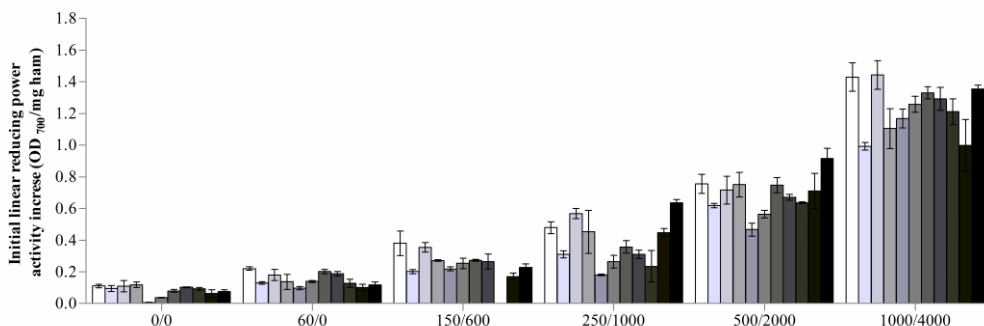
Figure 2 Reducing power per mg ham used for the extracts of the crude ≤ 10 kDa extracts over time.



480

481 Figure 3 Reducing power (OD_{700}) expressed per mg ham used for meat/water homogenate measure in the
 482 ≤ 10 kDa extract of the hams with different amounts of added sodium nitrite or sodium ascorbate measure
 483 after 0 days of storage (for 0/0, 60/240 and 150/600 $n=3$ while for 250/1000, 500/2000, 1000/4000 $n=6$).

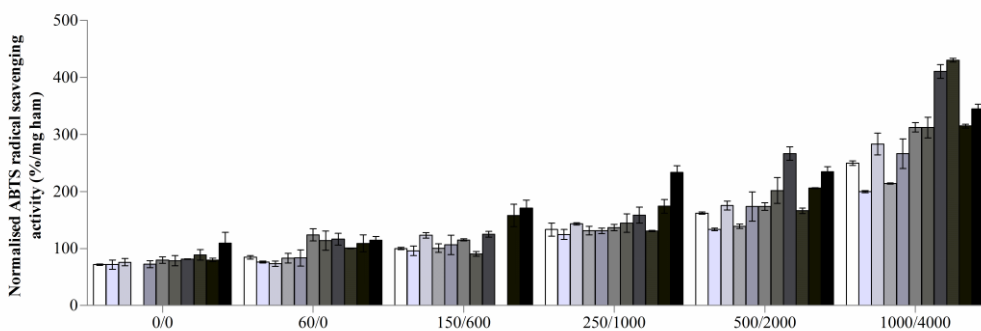
484



485

486 Figure 4 Average initial linear reducing power activity (%/mg ham) in the crude ≤ 10 kDa extracts during
 487 storage distributed on ham type. The white bar indicates 0 days of cold storage and for each shade darker +3
 488 days of storage until the black bar indicating 30 days of cold storage. Sample 150/600 at day 24 was lost.

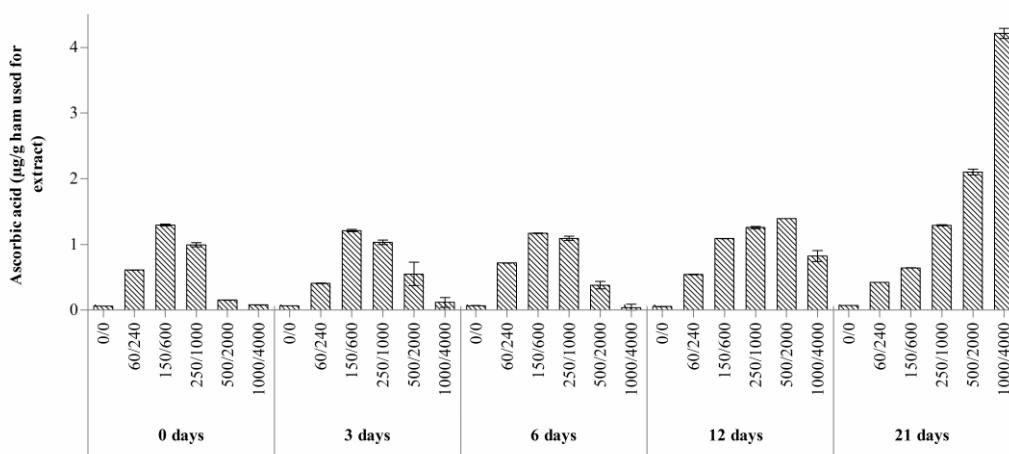
489



490

491 Figure 5 Normalised ABTS radical scavenging activity (%/mg ham) in the crude ≤ 10 kDa extracts during
 492 storage distributed on ham type. The white bar indicates 0 days of cold storage and for each shade darker +3
 493 days of storage until the black bar indicating 30 days of cold storage. Sample 0/0 day 9 and 150/600 at day
 494 24 were lost.

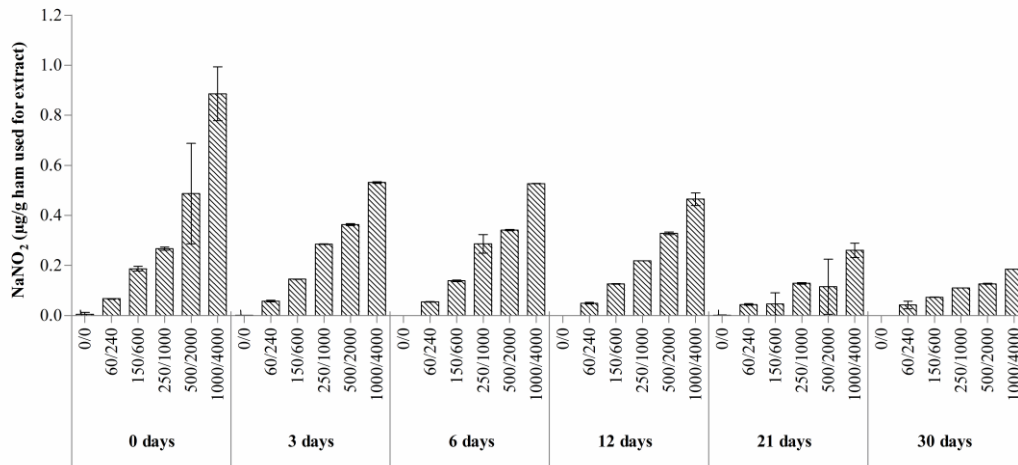
495



496

497 Figure 6 Average ascorbic acid content in the crude ≤ 10 kDa extracts made from hams at all concentration
 498 levels stored for 0, 3, 6, 12, 21 days at 5°C. The content has been normalised to the amount of ham utilised
 499 for the ham/water homogenate for producing the extracts. (n=2).

500



501

502 Figure 7 Average sodium nitrite content in the crude ≤ 10 kDa extracts made from hams at all concentration
 503 levels stored for 0, 3, 6, 12, 21 and 30 days at 5°C. The content has been normalised to the amount of ham
 504 utilised for the ham/water homogenate for producing the extracts. (n=2).

505

National Food Institute
Technical University of Denmark
Kemitorvet
Building 202
2800 Kgs. Lyngby
Denmark

T: 35 88 70 00
F: 35 88 70 01
www.food.dtu.dk